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1. Mintz, A. A.: Antibiot. Med. 7:481, 1960.

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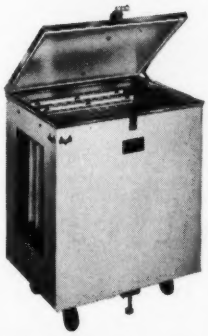

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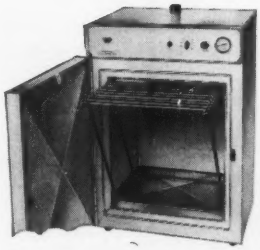

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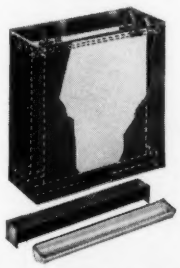

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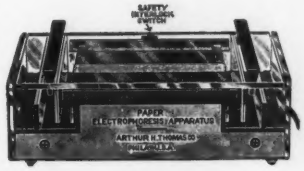

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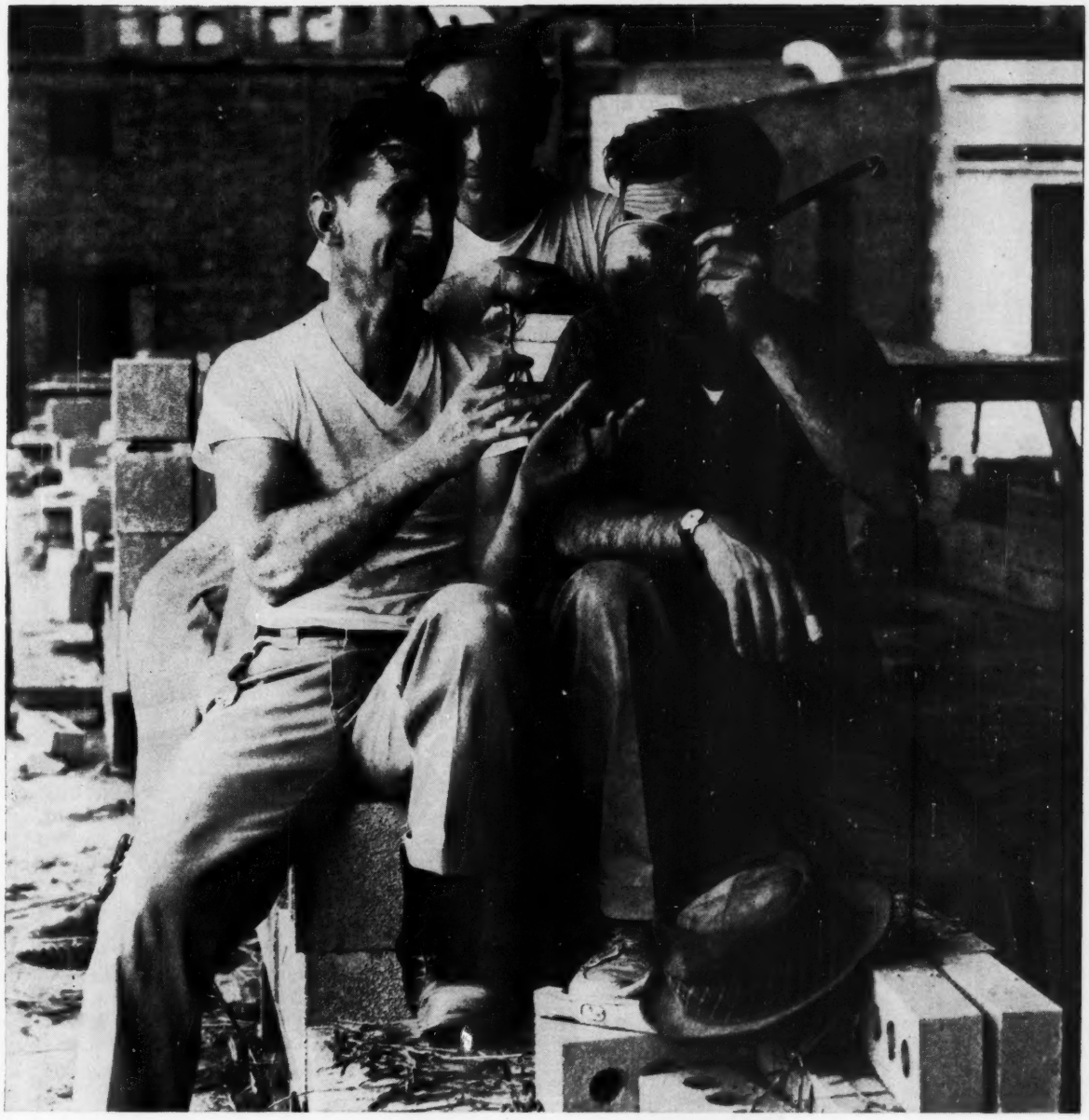

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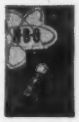

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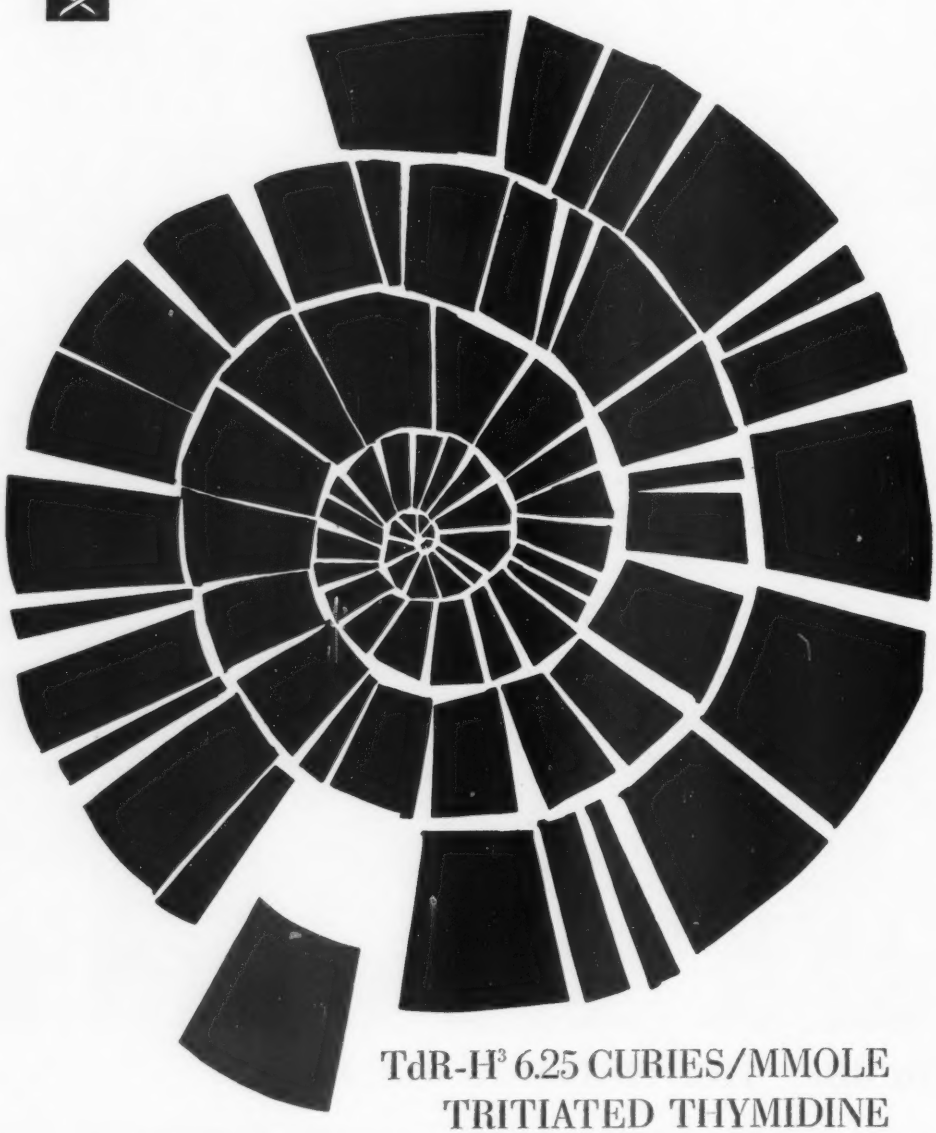

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Beginning in 1962, this journal will be published bi-monthly:

January-February

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May-June

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An index and volume contents will appear in the November-December issue.

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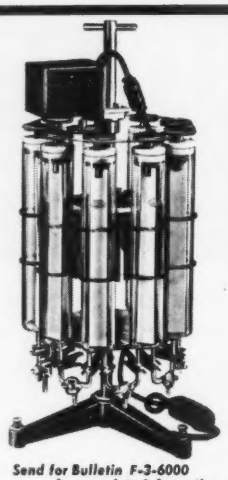

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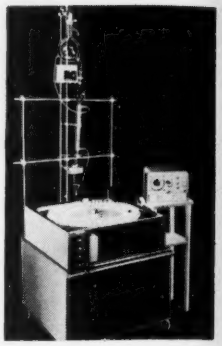

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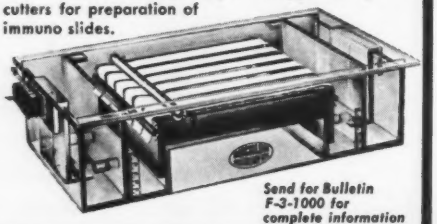

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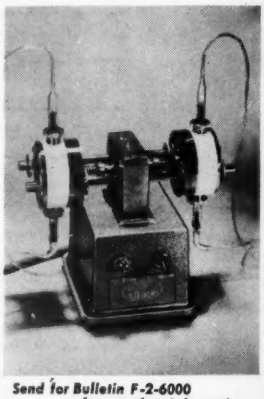

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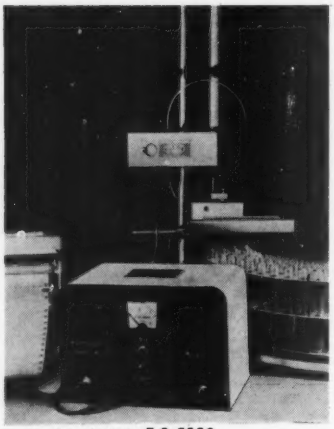

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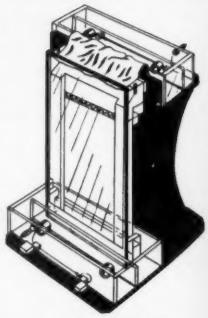

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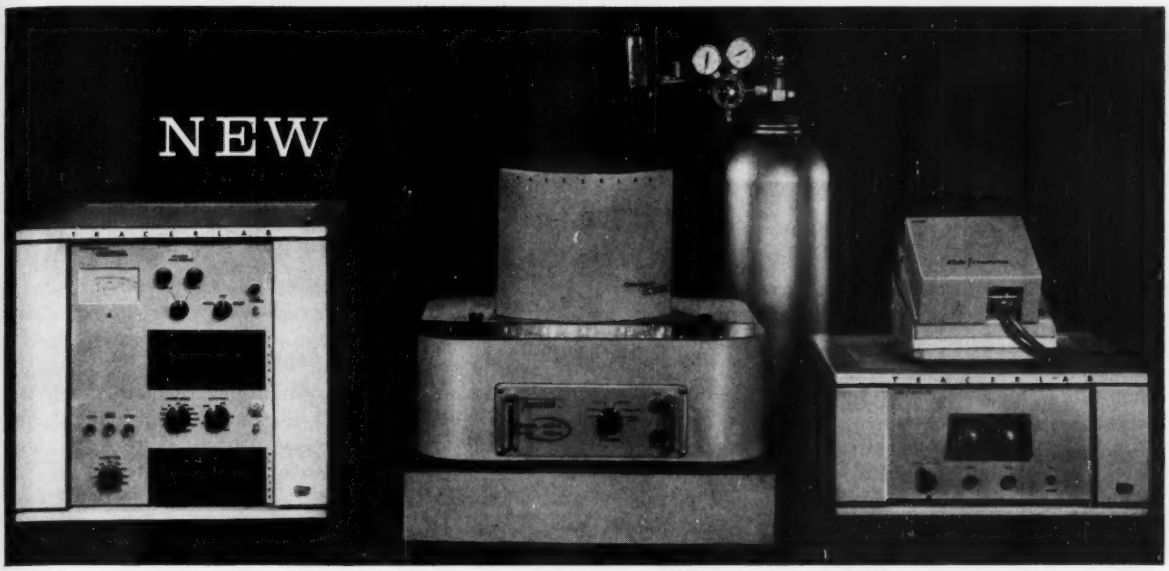

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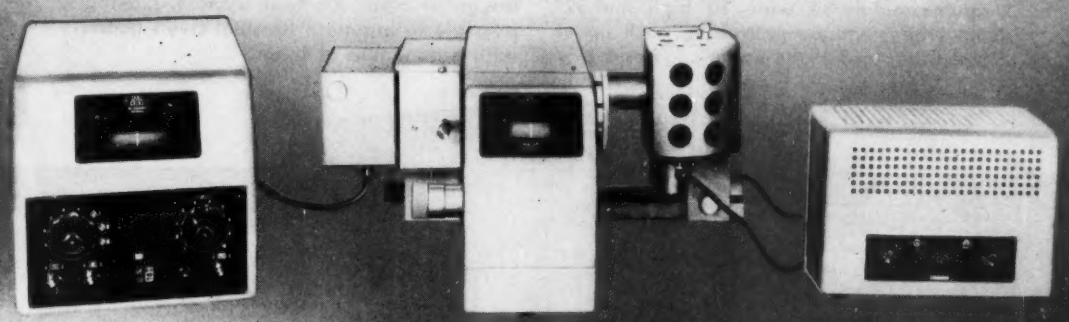

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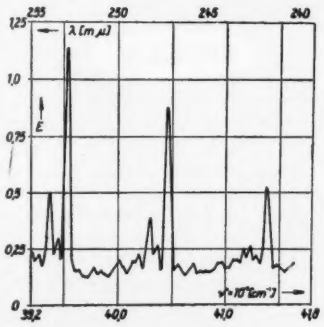

Extinction curve of benzine vapor.

Attachments available:

Fluorescence Attachment
Chromatogram Attachment
Reflectance Attachment
Micro Cell Attachment
Flame Equipment

The spectral region ranges from far-ultraviolet 200 mμ to near-infrared 1,000 mμ (2,500 mμ by using a lead sulphide cell). The instrument operates directly on 110-volt A.C. Voltage fluctuations of + 10% to − 15% can be compensated for by means of its combined magnetic and electronic voltage stabilizer. The transmission and extinction scales are enlarged and projected on a ground-glass window, assuring parallax-free readings.

The adjustment of the slit and that of the wave length is guaranteed to be free of lost motion. The slit width can be set within 0.2 mμ absolute. As a consequence of the friction-free design, no lost motion is evident when setting the wave length. For instance, the combined error of setting and reading the wave length is guaranteed to be no greater than ± 0.05 mμ at 250 mμ. The effective length of the projected micro wave length scale is enlarged so that it is equivalent to a scale approximately 4 feet long and has a range from 200 mμ to 2,500 mμ.

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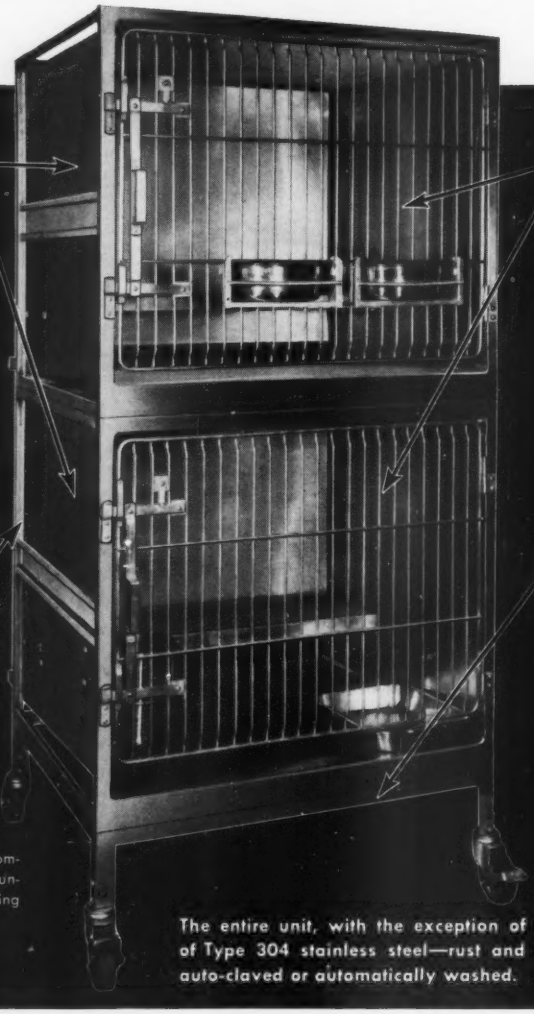
The ACS Mobile Unit consists of two cages mounted on a stainless steel frame. Each cage is approximately 34" wide, 30" high, and 32" deep, with a unique protruding back lip of 2½" on the floor of the upper cage and 1½" on the floor of the lower cage. This design feature allows cages to drain into a wall trough or other drainage system, making it possible to maintain hospital-clean quarters with a minimum of labor.



CAGE

Constructed of Type 304, 20-gauge stainless steel. The entire cage is seam welded into a one-piece unit. Each corner, wall-floor and wall-ceiling junction has a radius to prevent the accumulation of dirt and animal excretions. Bottom of each cage sloped from front to a 2" opening in back. Equipped with an oak resting board that provides comfort to the animal.

FRAME

Made of stainless steel angles, ¼" x 1" x 1", spot welded to front and sides of cage.

GATE

Made of ½" stainless steel rods vertically spaced 1¼" clear between rods, with ⅜" stainless steel rod frame. ACS stainless steel latch and handle. Gate is removable for easy cleaning. Choice of two feeding arrangements: (1) Waterer on side of cage, with feeding pan inserted in bottom of gate. (2) Two pans inserted in the gate proper.

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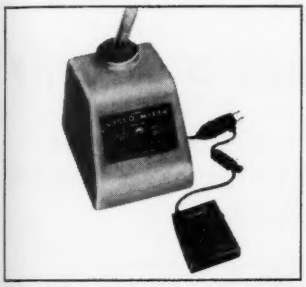

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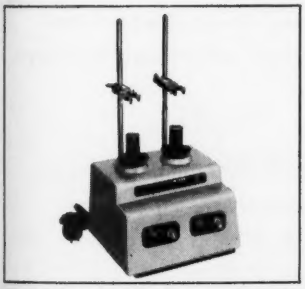

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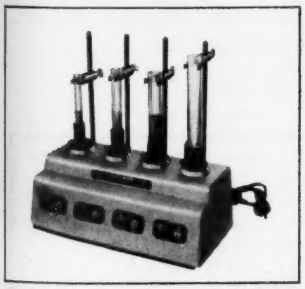

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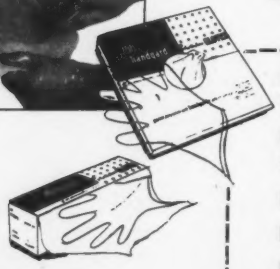

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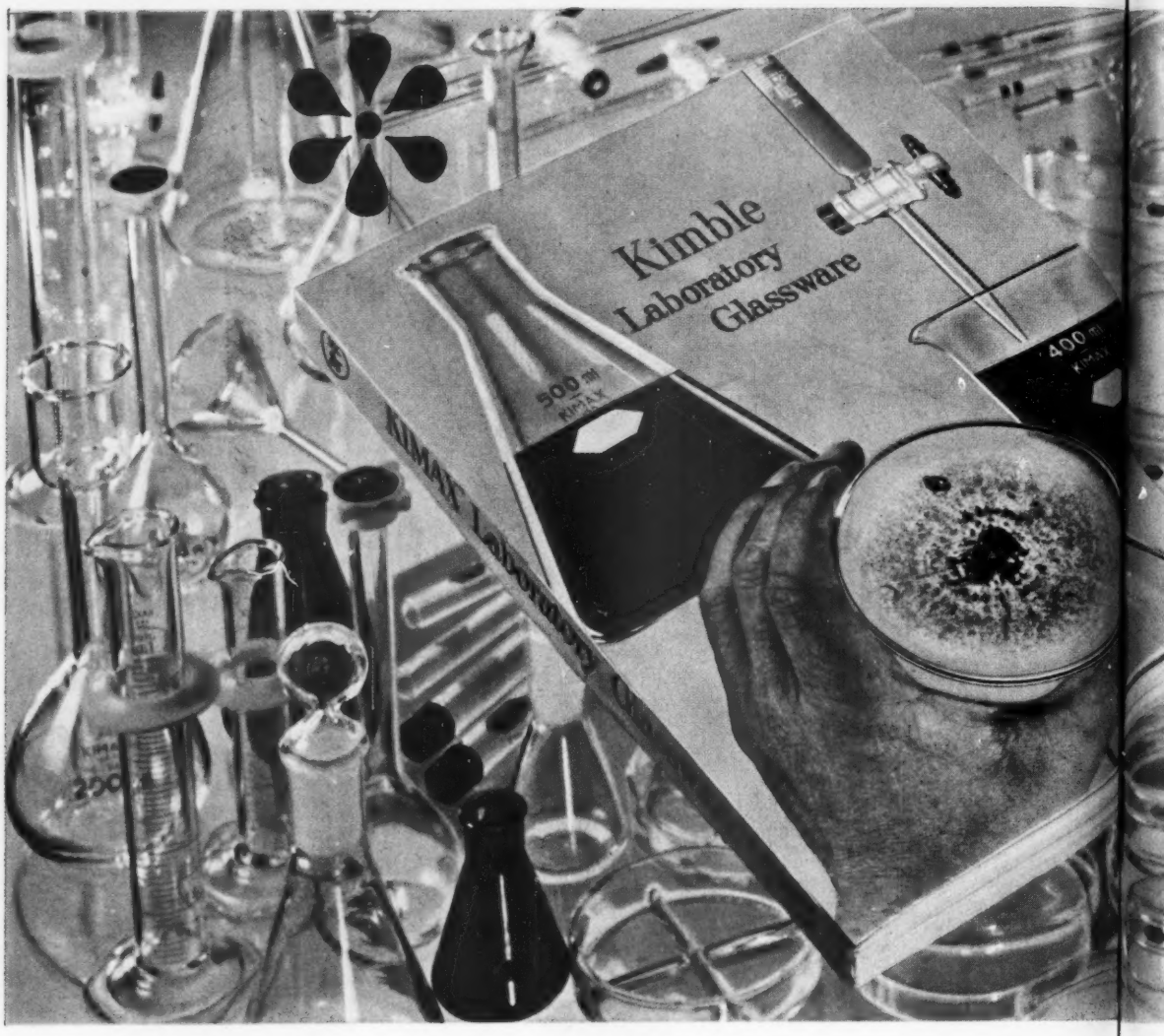

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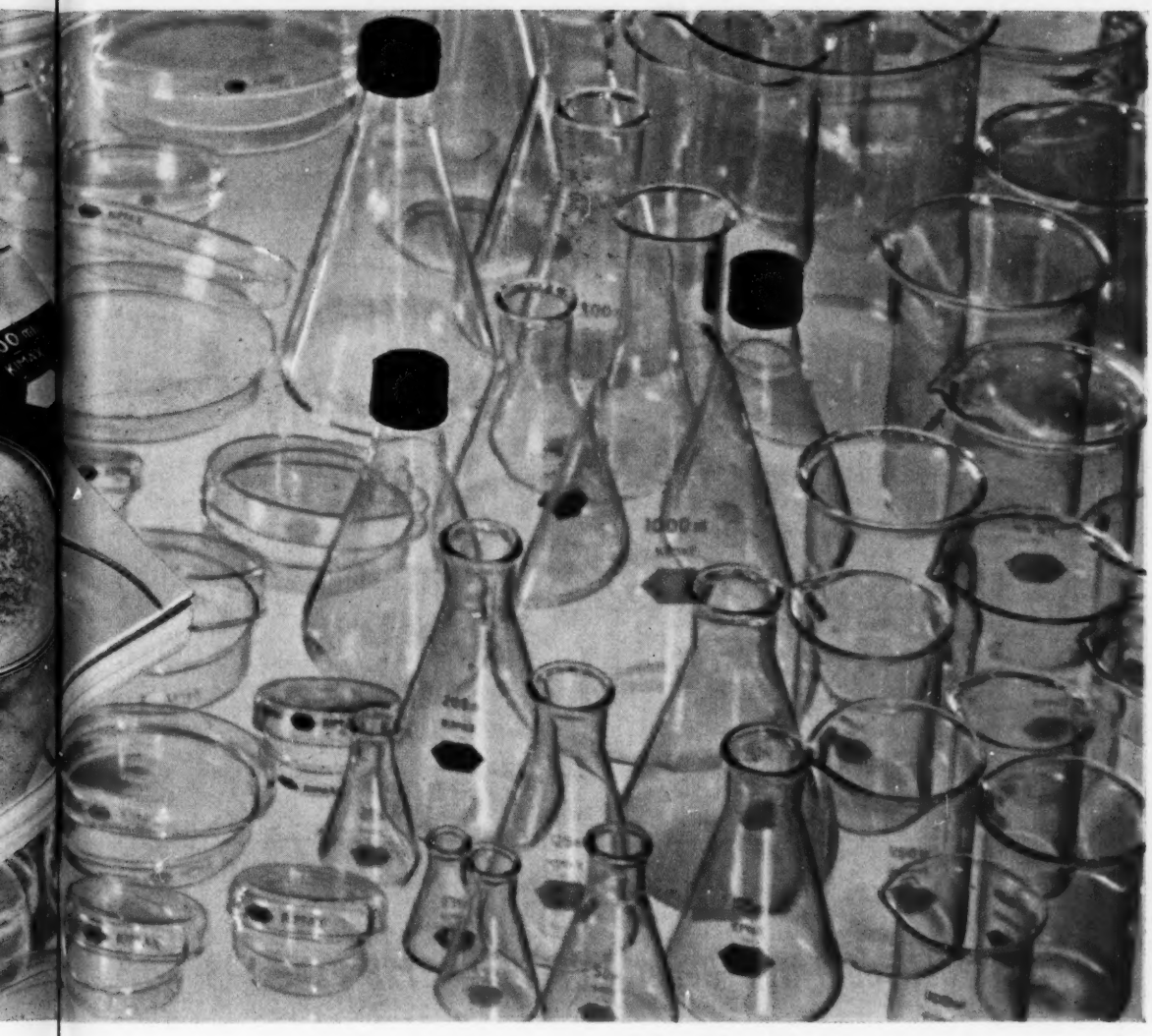

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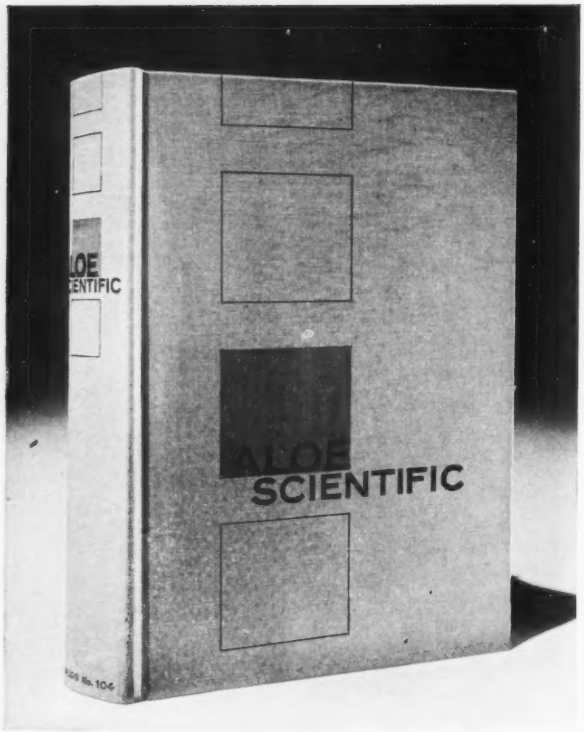

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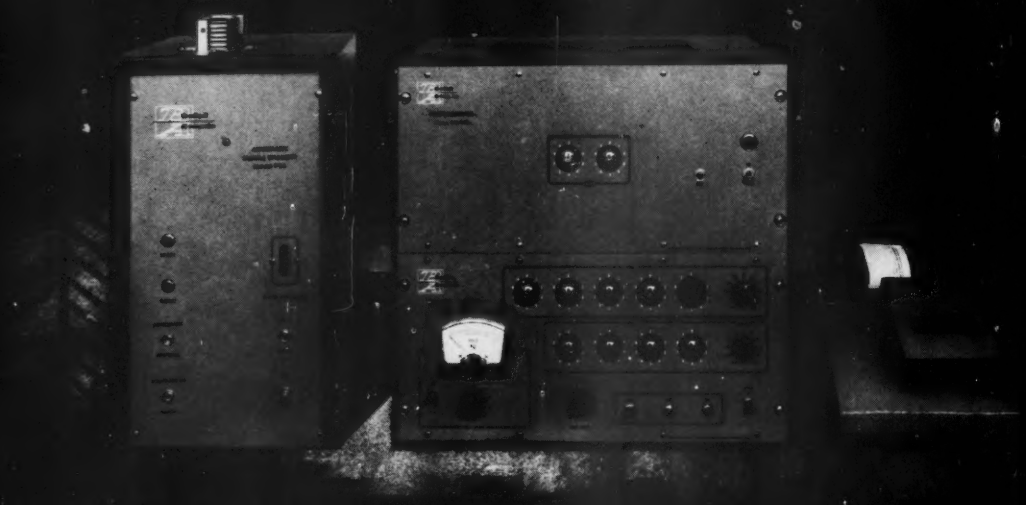

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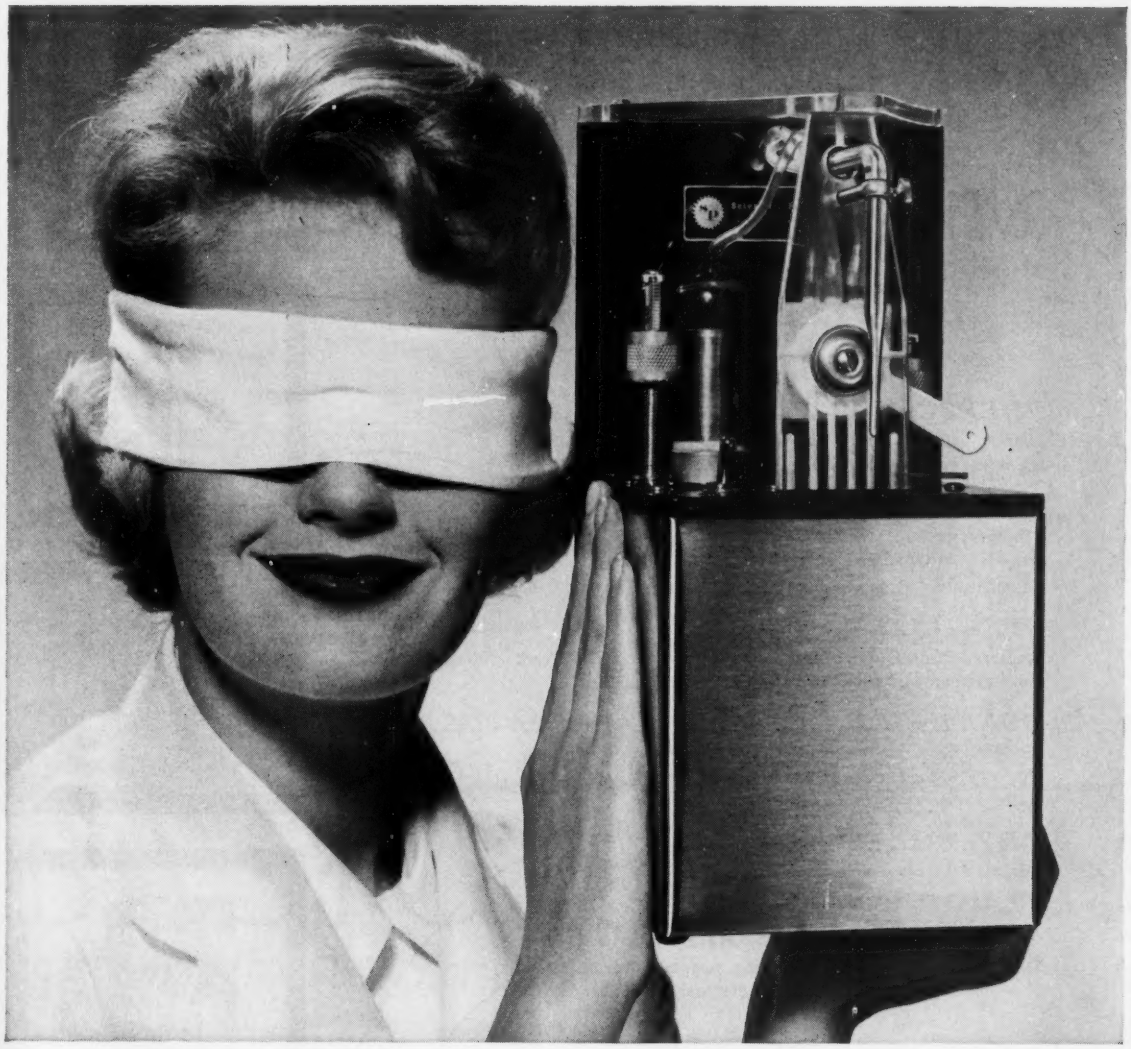

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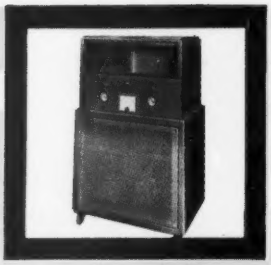

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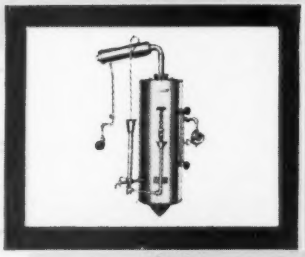

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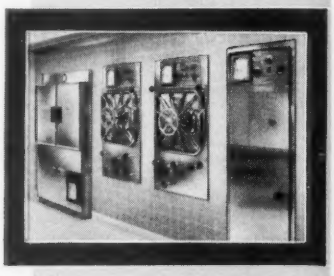

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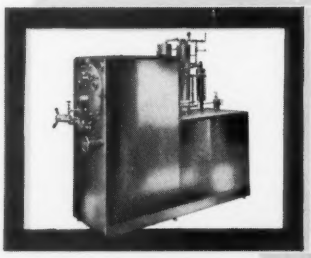

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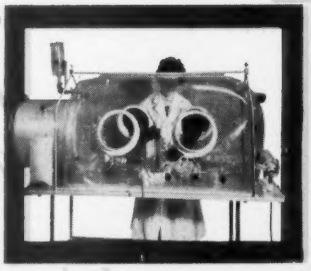

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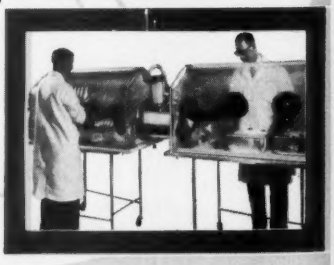

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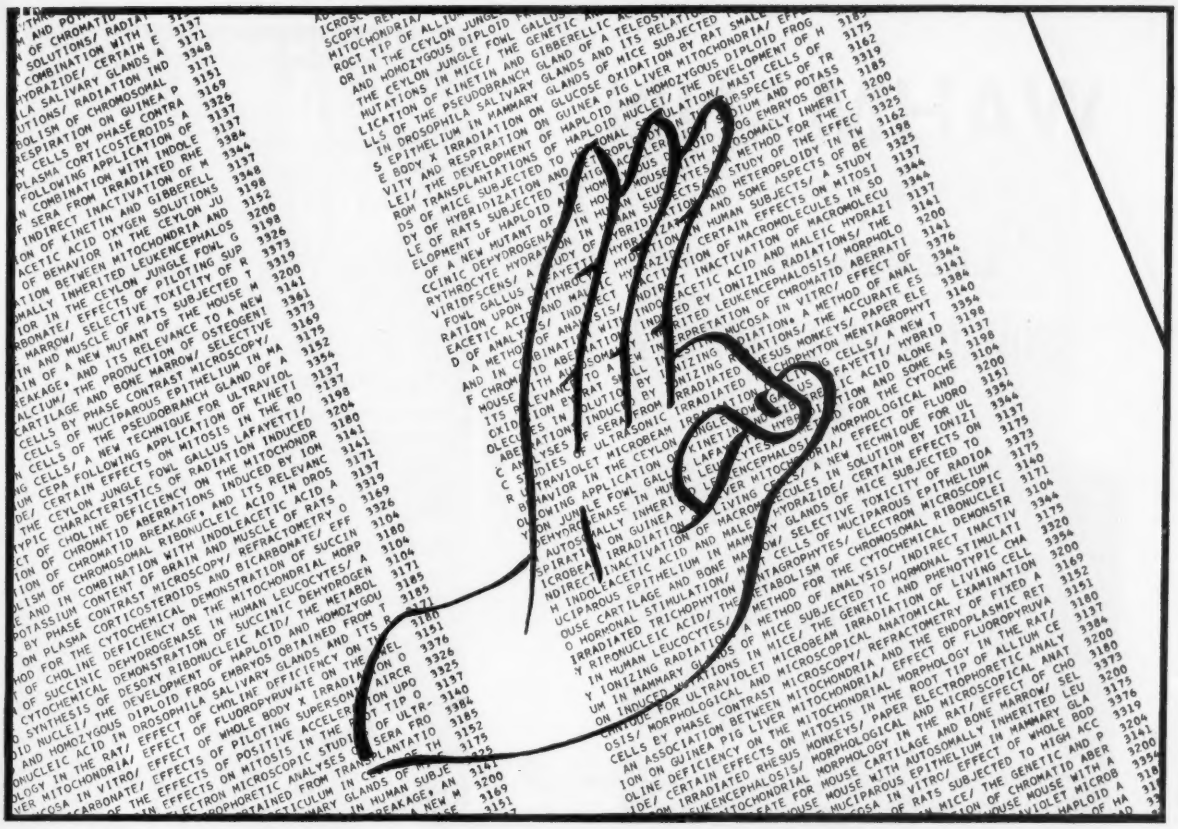

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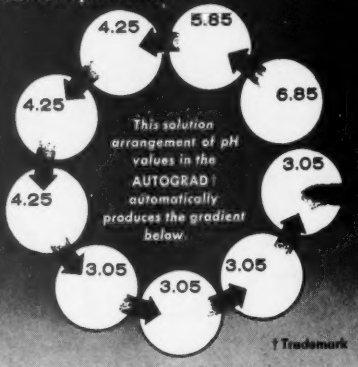

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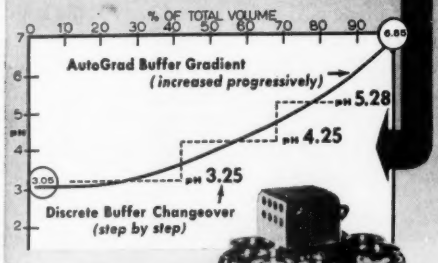

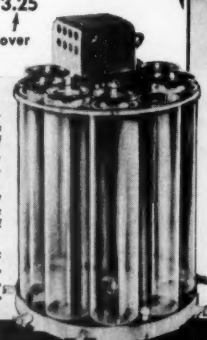

Developed at the National Cancer Institute, National Institutes of Health, U. S. Department of Health, Education, and Welfare, Public Health Service, Bethesda, Maryland.

***Procedure developed by *Karl A. Piez, National Institute of Dental Research, National Institute of Health.*

*Reference: *"Variable Gradient Device for Chromatography", E. A. Peterson and H. A. Sober, Anal. Chem., Vol. 31, No. 34, May 1959.*

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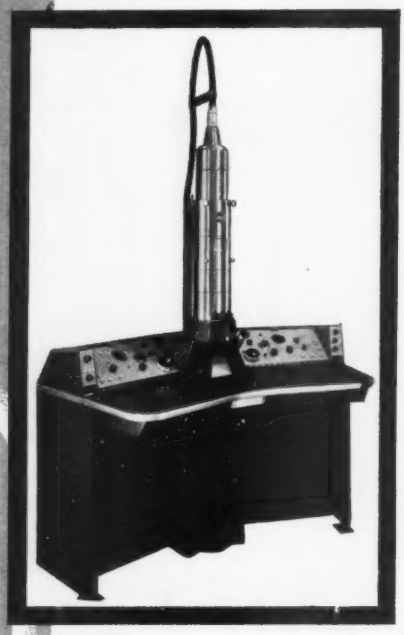

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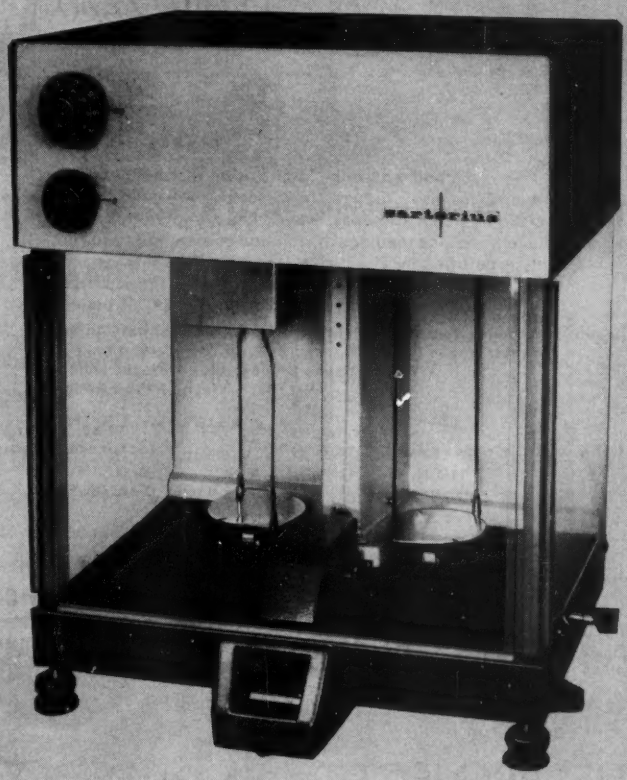

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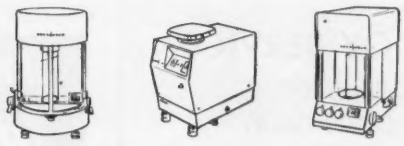

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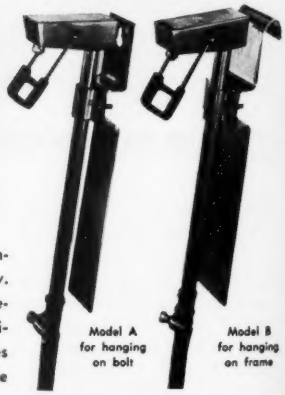

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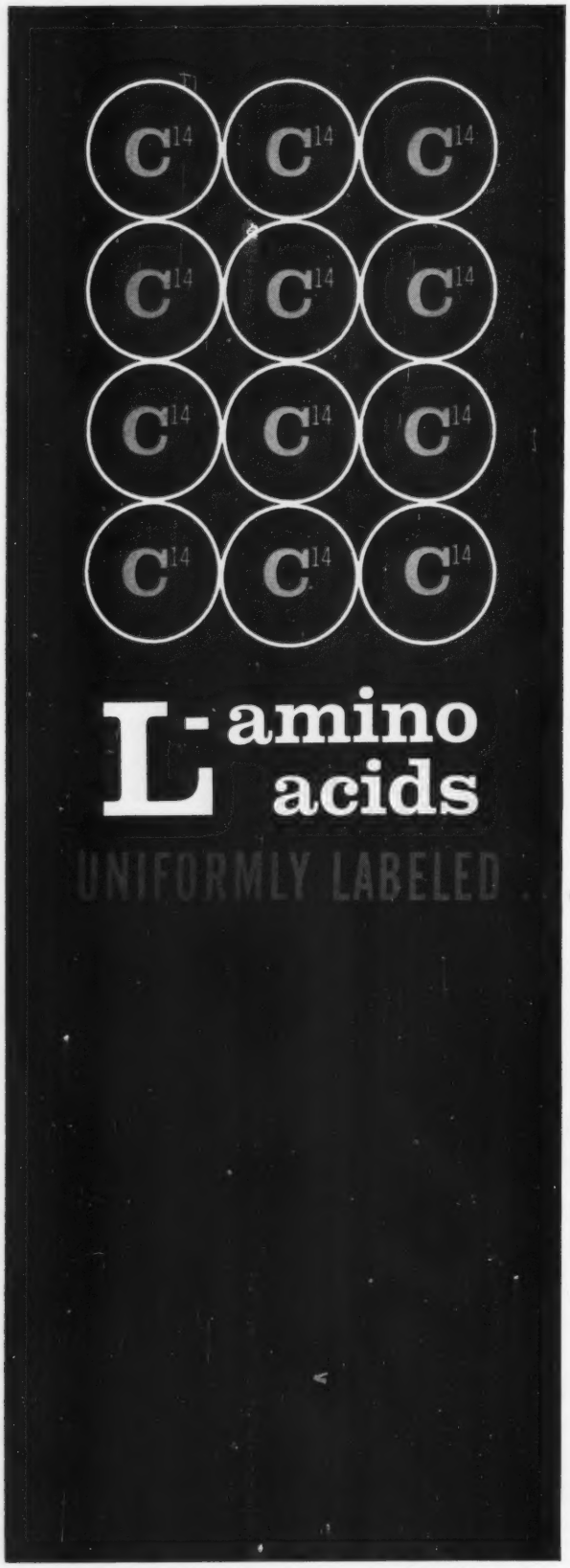

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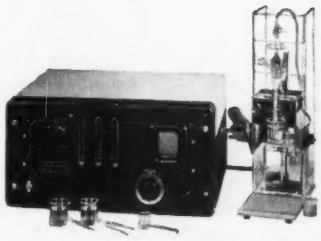

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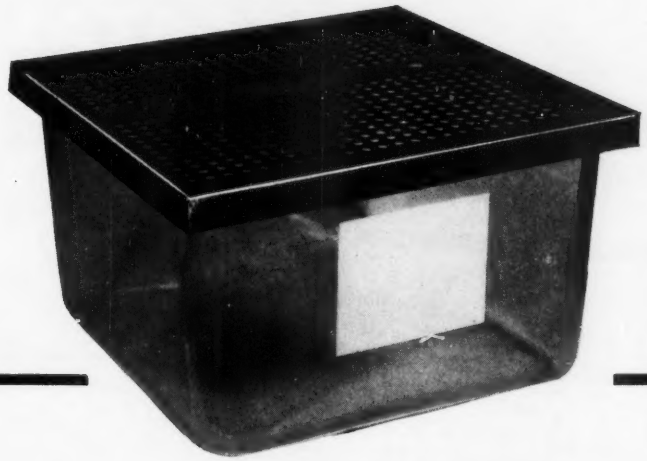

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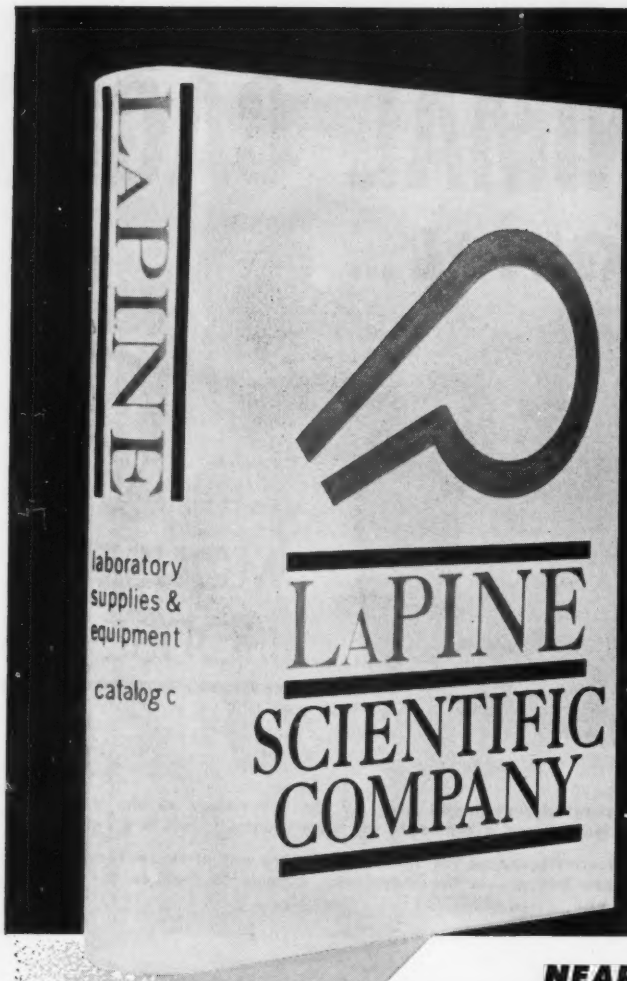

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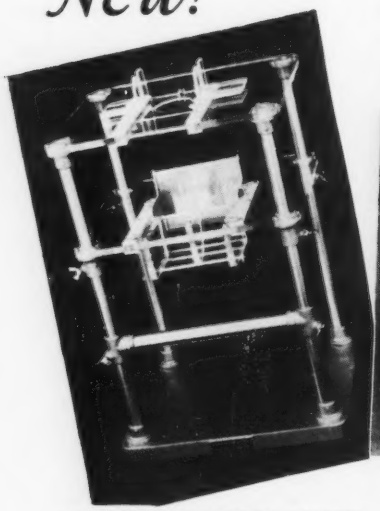

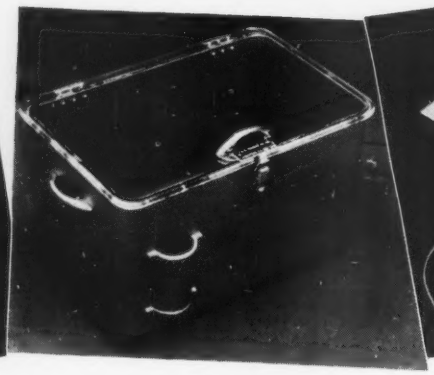

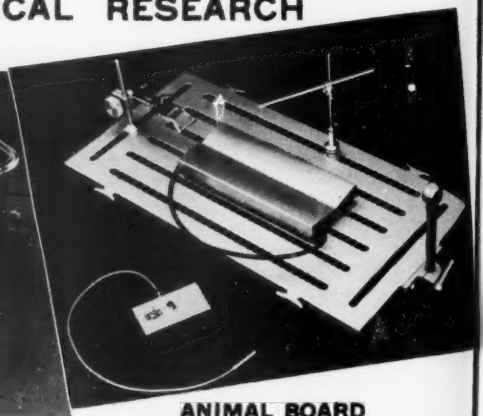

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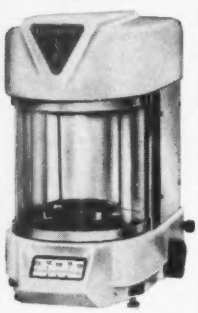

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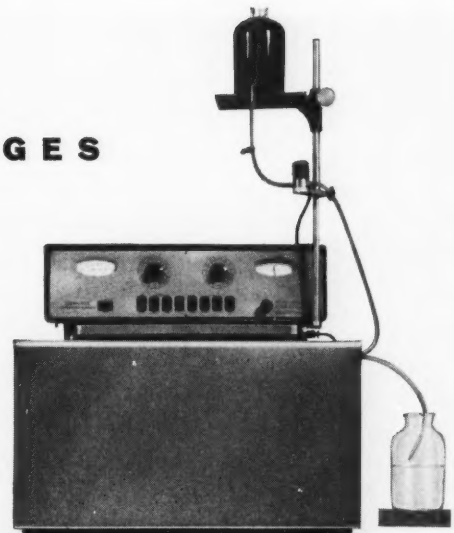

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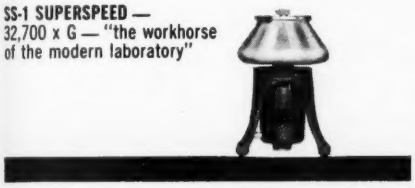

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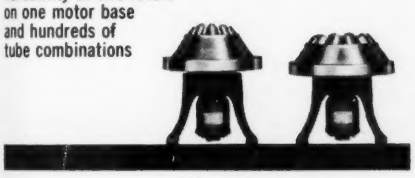

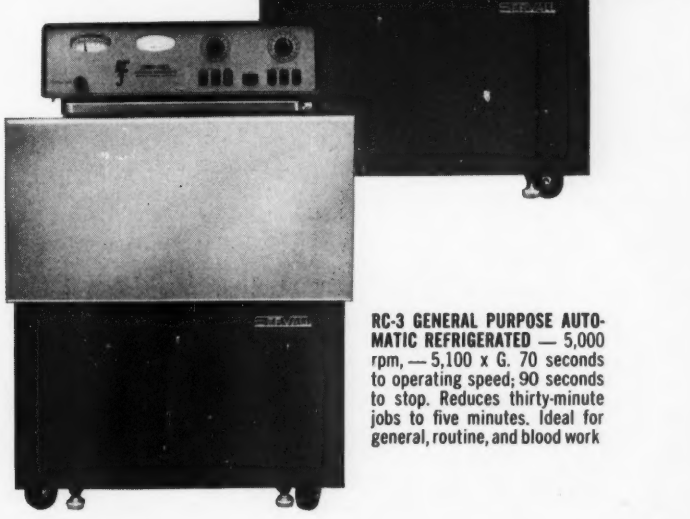

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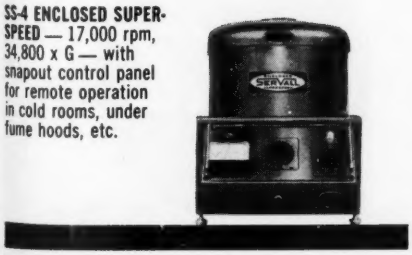

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Ludwik Gross, M.D., F.A.C.P., *Chief, Cancer Research Unit, Veterans Administration Hospital, Bronx, N. Y.; Associate Scientist, Sloan-Kettering Institute*

an important contribution to the data on viral tumors . . .

In this significant book, the author makes an extensive review of the known oncogenic viruses and discusses the current status of experimental research on virus-caused neoplasms.

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partial contents:

Introduction; Experimental inoculation of communicable diseases; Transplantation of tumors in animals by cell-graft; Transmission of tumors by filtrates; Rabbit myxomatosis; Rabbit fibroma; The transmissible fibroma of grey squirrel; The Shope rabbit papilloma; Oral papillomatosis of dogs; Warts in cattle; Transmission of human warts and papillomas by filtrates; The Lucké frog kidney carcinoma; The contagious veneral dog sarcoma; The Rous chicken sarcoma; The chicken leukosis complex; Avian myeloblastosis; Avian lymphomatosis and related tumors; Development of inbred strains of mice and their impact on experimental cancer research; Mouse mammary carcinoma; Mouse leukemia; Radiation-induced leukemia in mice; Induction of leukemia in mice following inoculation of filtrates from mouse tumors; The parotid tumor (polyoma) virus; The search for oncogenic viruses in humans.

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Editors:* S. S. Key and J. Elkes, *National Institute of Mental Health, Bethesda

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contents

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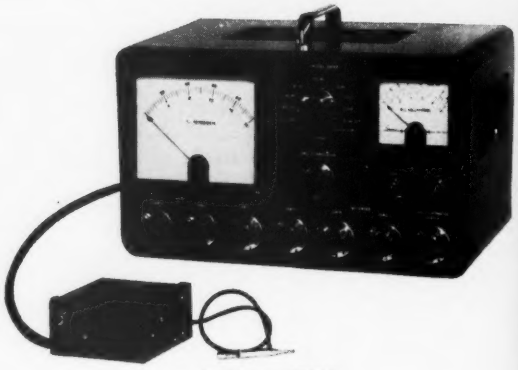

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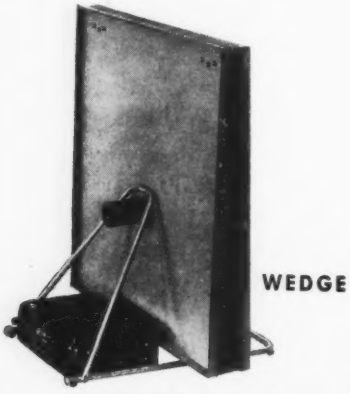

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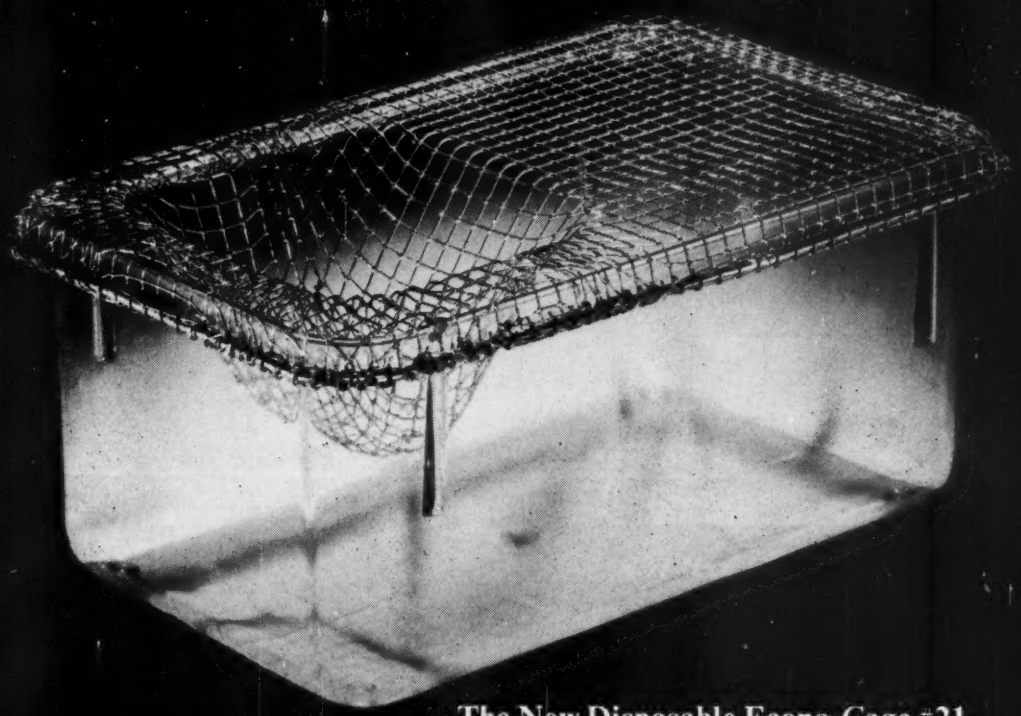

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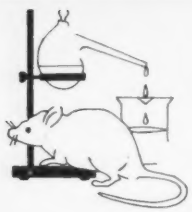

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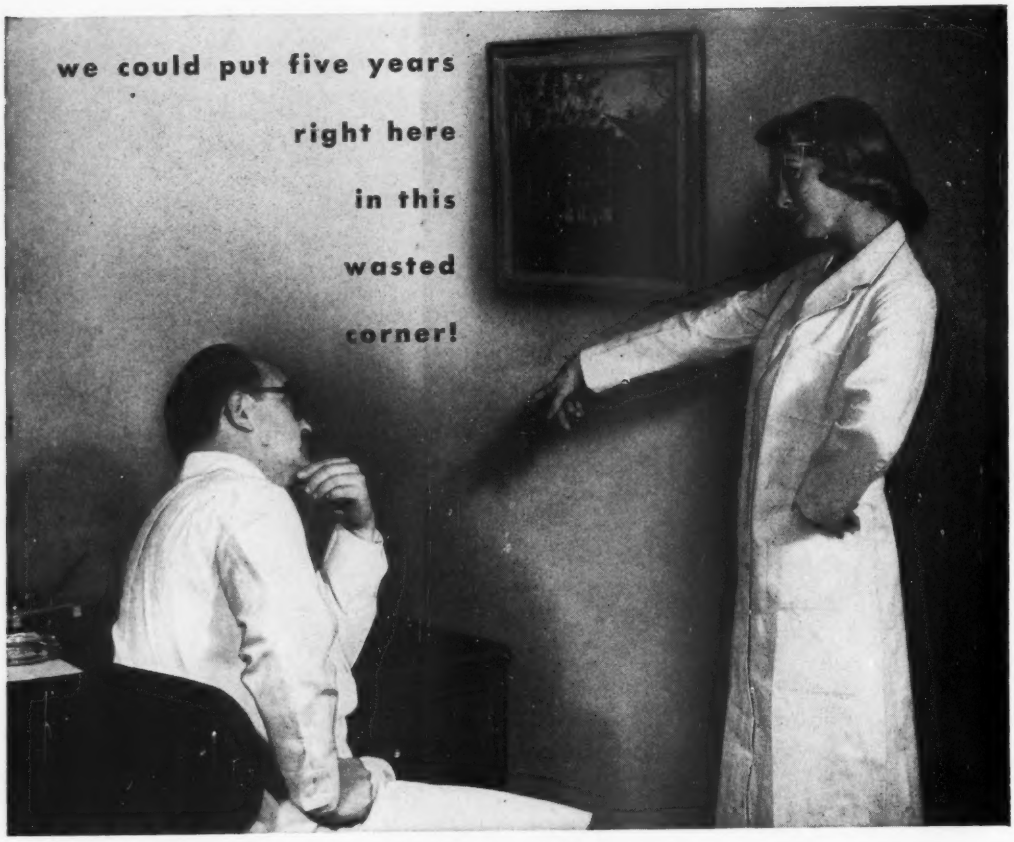

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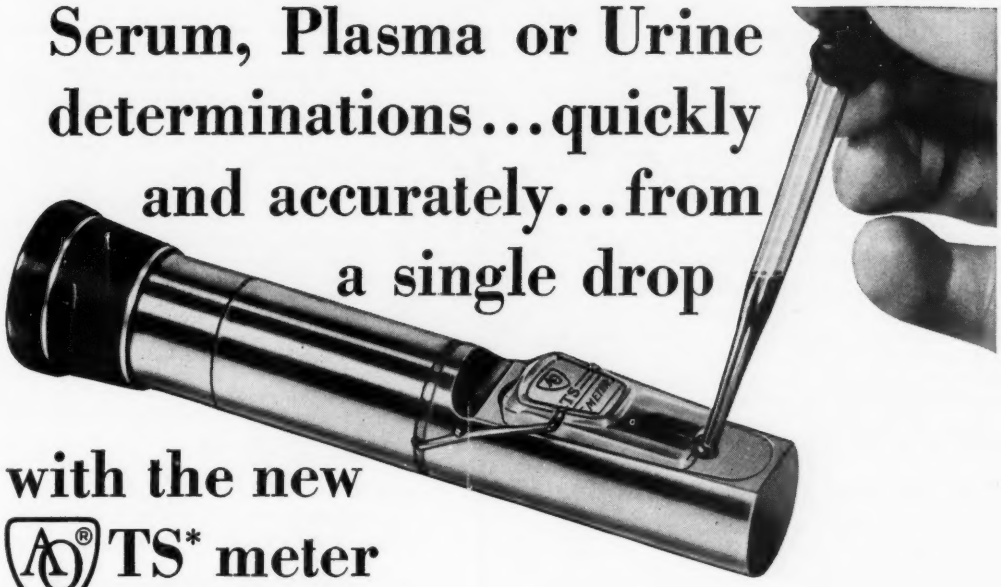

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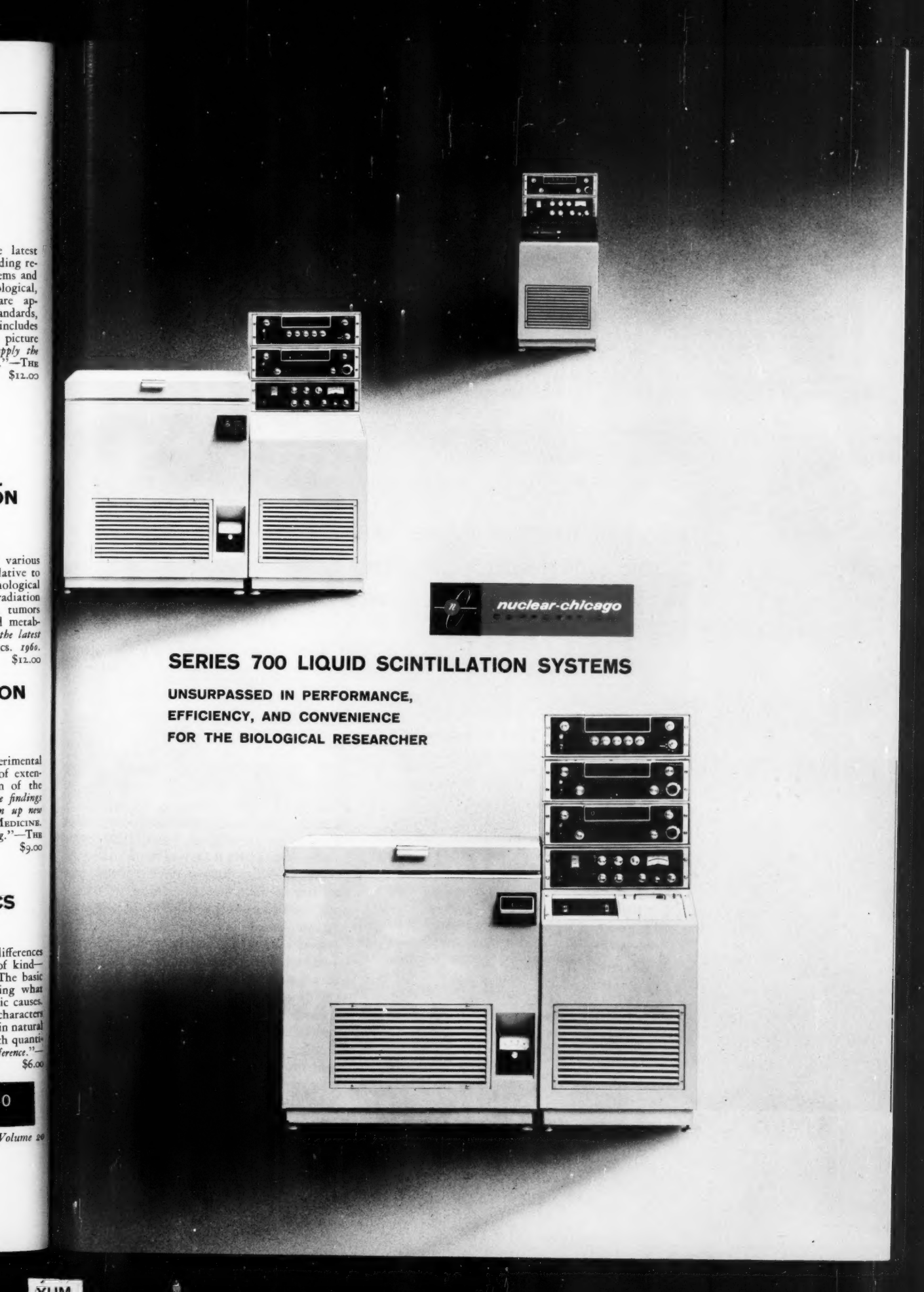
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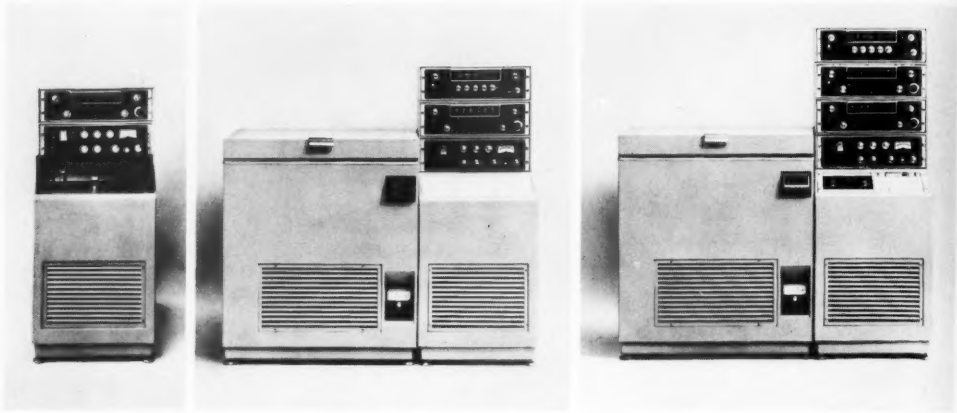

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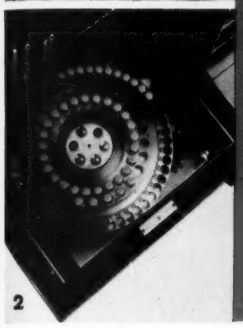
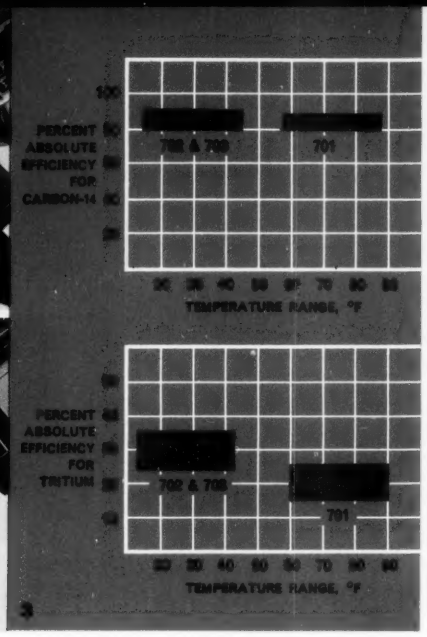




1) Sample being placed on elevator of detector assembly in 702 controlled temperature chamber. Ample space is provided in cooling chamber for storage of samples.

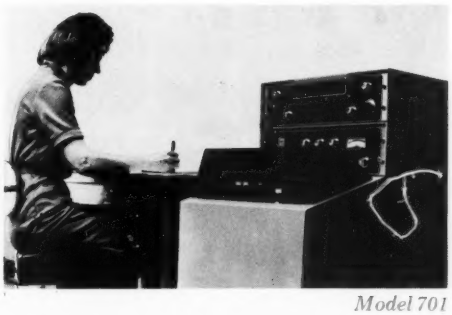
2) Automatic sample changer in the 703 cooling chamber. Sample bottles are located at the edge of the circular table. Removable racks for additional samples and scintillation standards are provided.

3) Charts of 700 Series efficiency ranges obtained in integral counting. Sample used was labeled toluene with PPO and POPOP. For carbon-14 the 702 and 703 yield efficiencies of 80-90%, while the 701 provides an efficiency range of 80-85%. For tritium the 702 and 703 provide an efficiency range of 25-35%, while the 701 efficiency is 15-25%.

in ordinary room lighting. Output signals from the solid state circuitry are shaped pulses which can feed any standard scaling instrument. Nuclear-Chicago's Model 8250 Logic scaler is shown here mounted atop the 701 console.

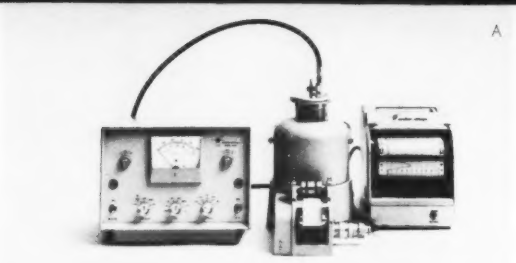
MODEL 702 has the dual-detector assembly mounted in a custom designed cooling chamber which permits highest efficiency for manual counting of C-14 and H-3 samples. The cooling chamber temperature is variable from +10° to +50°F and is stable with ±1°F. A pushbutton control on the front of the cooling chamber operates the motor-driven elevator which positions the sample in the dual detector assembly. Logic 8250 scaler and Logic 8600 timer are shown.

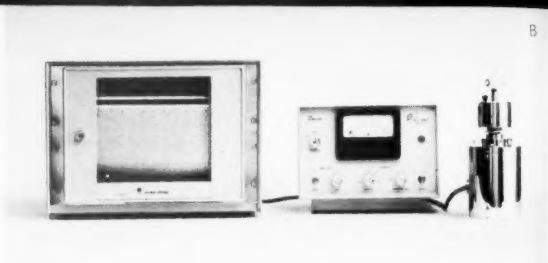
MODEL 703 is the most advanced counting system of its kind, providing both automatic sample changing and temperature controlled operation. Its sophisticated options of data presentation and versatile modes of automatic operation suggest it as the system of choice for laboratories counting many samples. A two-digit display on the cooling chamber shows the number of the sample which is in the counting position. The changer accepts up to 50 samples at a time, and an additional removable rack holds 50 more samples for temperature adaptation prior to measurement. Two 8250 scalers, 8600 timer, and 8401 digital recorder are shown.

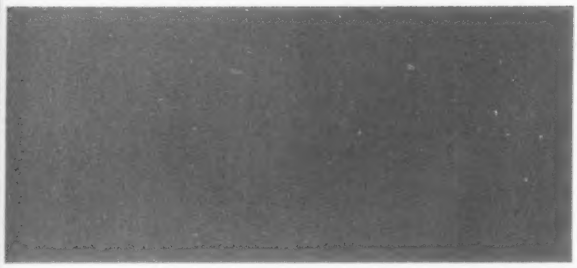


Model 701

Model 703







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The systems presented here demonstrate the scope and completeness of Nuclear-Chicago's products for the biological researcher. They are—instrument by instrument, and system by system—capable of efficient, precise performance with unusual reliability. They are representative of the more than 250 instruments, counting systems, radionuclides and accessories presented in detail in Nuclear-Chicago's new, 96-page general catalog. Please send for your copy — you will find unequalled variety and versatility in the complete Nuclear-Chicago line.

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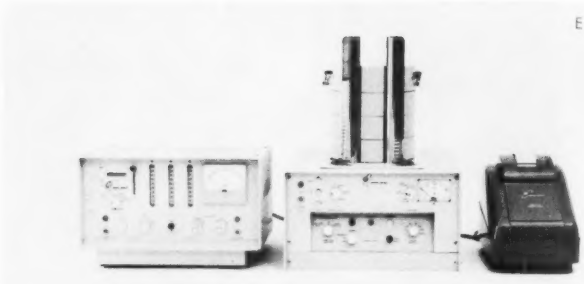
B. 6000 Dynacon® Electrometer measures beta radiation in solid, liquid, or gaseous samples.

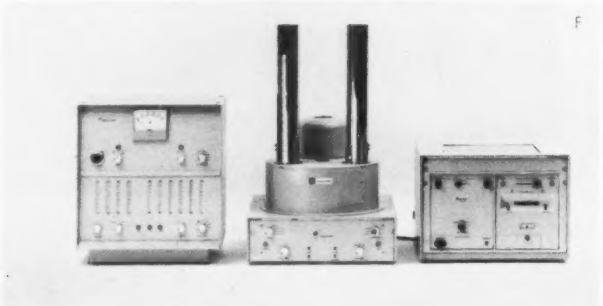
C. C120 Automatic Sample Changer accepts up to 49 gamma-emitting samples in test tubes. Scintillation well detector is mounted in console.

D. C110B Automatic Sample Changer accepts up to 35 solid beta samples 1 in. or 1¼ in. diameter.



E. C115 Automatic Beta Sample Changer is supplied with guarded gas-flow detector with background of only 2 cpm.

F. C210 Automatic Sample Changer accepts 50 or more beta samples up to 2 in. diameter.



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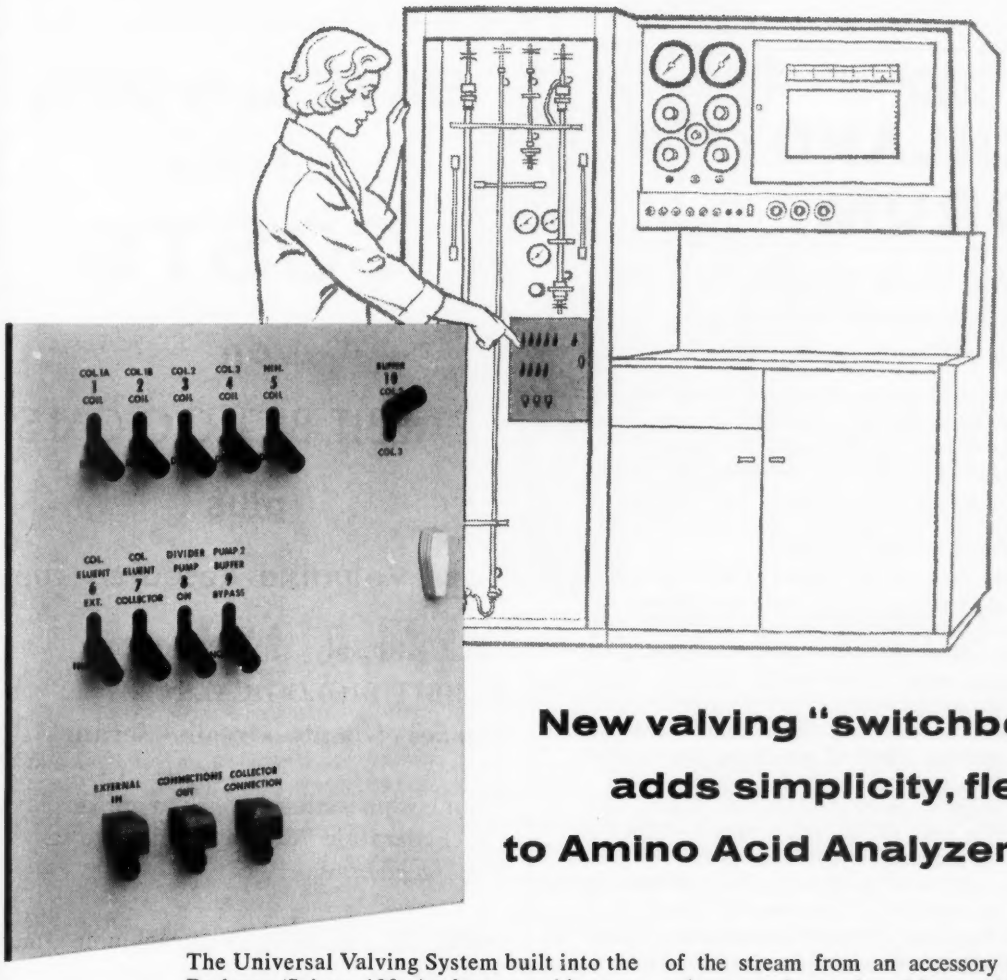

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The Universal Valving System built into the Beckman/Spinco 120 Analyzer provides two key advantages to instrument owners:

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For complete information on the 120 Amino Acid Analyzer, write Beckman Instruments, Inc., Spinco Division, Stanford Industrial Park, Palo Alto, California. Ask for Data File 120-6

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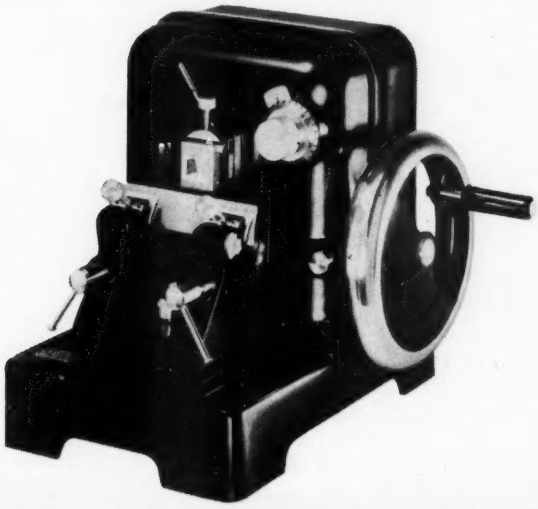

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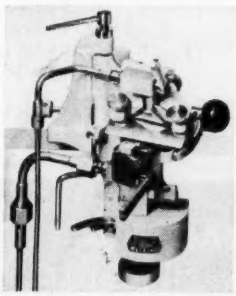

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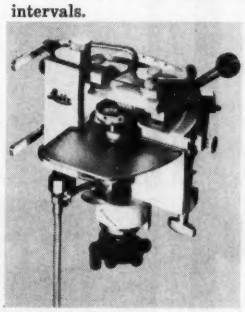

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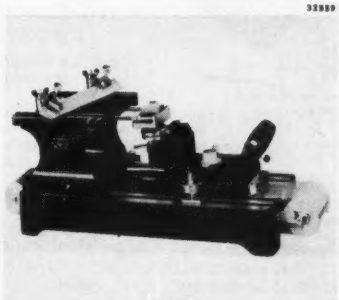

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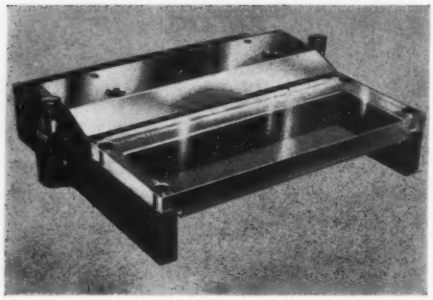

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* *G. Majno and W. E. Bunker. Preparation of Tissue Slices for Metabolic Studies: A Hand Microtome Especially Suitable for Brain. Journal of Neurochemistry, 1959, Vol. 2, Pages 11-14.*

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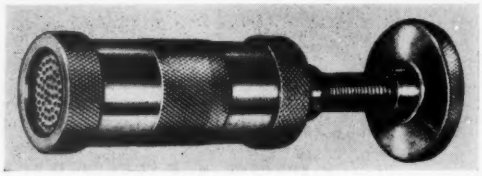

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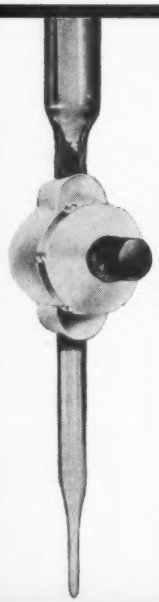

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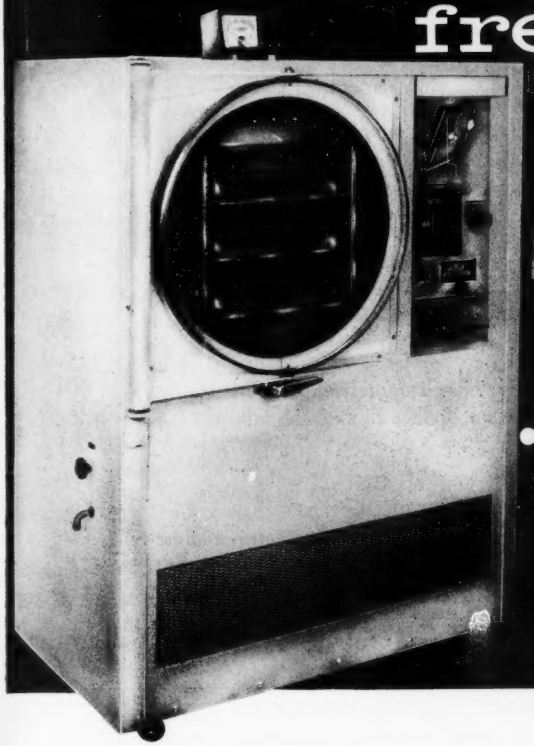

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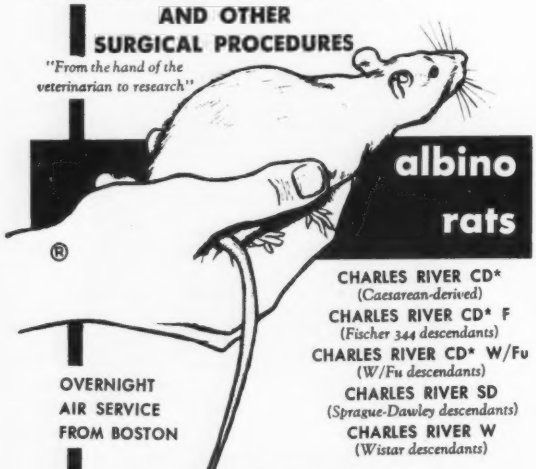

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THE FOLLOWING HOURS WILL BE OBSERVED:

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Advance registration will be accepted at the Washington Office of the Federation until March 30, 1962. Employers using the interview facilities must be either annual subscribers to the Placement Service Lists or subscribe to the February 1962 List of Candidates.

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3. Publishes ANNUALLY a list of fellowships, assistantships and traineeships
4. Provides registration and interview facilities for candidates and employers at the annual meeting.

The following scale of charges has been authorized by the Federation Board:

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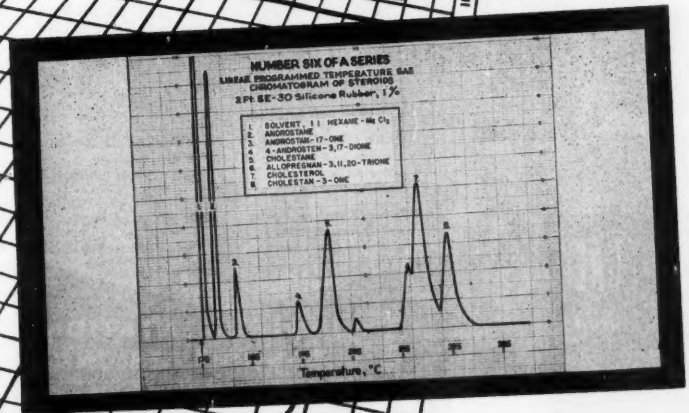

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The chromatogram at the left was obtained on F & M's Model 609 Flame Ionization Linear Programmed Temperature Gas Chromatograph, using a 2 ft. column of 1% silicone gum rubber programmed from 175° to 235°C. Note that androstane was separated from the solvent and all the peaks have similar shape for easy analytical measurement. The same instrumentation and column are also adaptable to sterols, cholesterol esters, bile acids and alkaloids.[3]

LITERATURE CITED

(1.) Beerthuis, R. K., and Recourt, J. H., *Nature*, 186, 372-374 (1960).

(2.) *Martin, A. J., Euston, C. B., and Martinez, F. W., Jr., "The Design and Evaluation of a Linear Programmed Temperature Gas Chromatograph Featuring a Hydrogen Flame Ionization Detector," presented at the Detroit Anachem Conference. October 1960.

(3.) VandenHeuvel, W. J. A., and Horning, E. C., *Biochem. Biophys. Research Commun.*, 3, 356-360 (1960).

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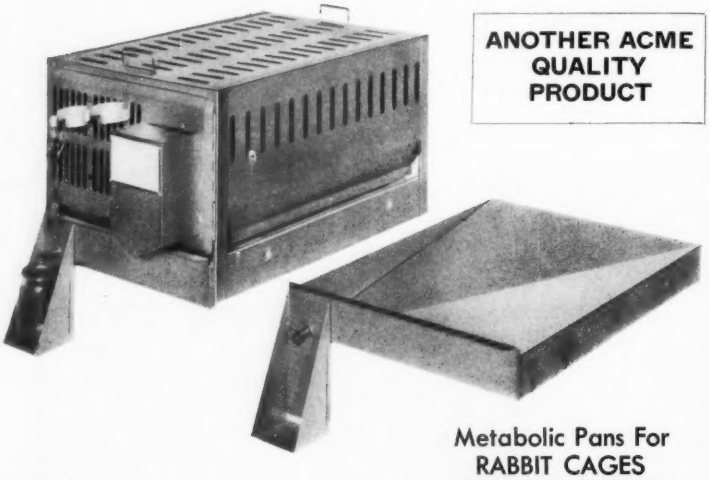

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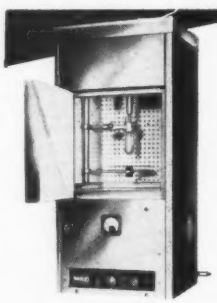

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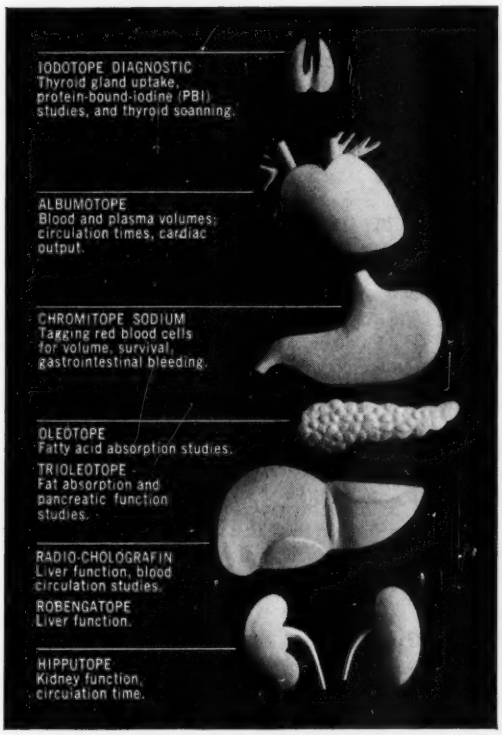

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News & Notes

FORTY-SIXTH ANNUAL MEETING
Atlantic City, New Jersey
April 14–19, 1962

The 46th Annual Meeting of the Federation will be held in Atlantic City, SATURDAY, APRIL 14, THROUGH THURSDAY, APRIL 19, 1962. The days of the week on which scientific sessions will be held have been changed to avoid conflict with the Easter weekend when Atlantic City hotels are heavily booked Because of the volume of papers to be presented, sessions will start at 9 a.m., Saturday, April 14, and *will continue for six days through Thursday afternoon*, April 19. Sessions will be held on Sunday, April 15.

Federation Headquarters, the Placement Service, exhibits, continuous motion pictures, and many of the scientific sessions will be in Convention Hall. Council rooms and some of the scientific sessions of the Societies will be located in the following headquarters hotels: Ambassador (Physiology), Dennis (Pharmacology), Ritz-Carlton (Biochemistry), Shelburne (Nutrition), Traymore (Pathology and Immunology). As in past years, there will be intersociety sessions and intersociety symposia.

The Federation Joint Session will be held in Convention Hall on Monday evening, April 16. Biochemistry mixers are scheduled for Saturday evening, April 14; Pharmacology, Pathology, and Nutrition dinners, and the Immunology mixer will be held on Tuesday evening, April 17.

Motion pictures will be shown continuously, theater style, from 9 a.m. to 5 p.m., during several days of the Meeting. Continuous film showings, inaugurated last year, were so favorably received that they will again replace the former Monday evening motion picture session.

Motion pictures may *not* be shown in the regular scientific sessions.

A visible directory of registrants, an information desk, a message center for distribution of mail and personal messages, and ticket sales for special functions will all be located in the Lobby, Convention Hall.

REGISTRATION INFORMATION

The registration area will be in the Lobby, Convention Hall. Hours are 12 noon to 11 p.m., Friday, April 13; 8 a.m. to 5 p.m., Saturday through Wednesday, April 14–18; and 8 a.m. to 3 p.m. on Thursday, April 19.

The regular registration fee for the 1962 Meeting is $15.00. However, students who *register in advance* will be allowed a reduced registration fee of $10.00. (Students who register in Atlantic City must pay the regular fee of $15.00.) To obtain the reduced rate the student must return, with his advance registration card and remittance, a statement signed by the Head of his Department certifying that the applicant is presently enrolled as a student in one of the fields represented by the Federation. Certificates will be sent on request from the Federation Convention Office.

As a time-saver in Atlantic City, advance registration is strongly urged for all members of the Federated Societies and non-member biologists, physicians and others who plan to attend the Meeting. Advance registration forms will be mailed on request from the Federation Convention Office.

Continued on page 66a

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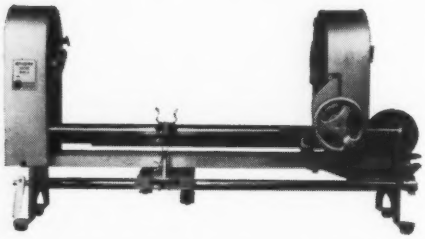

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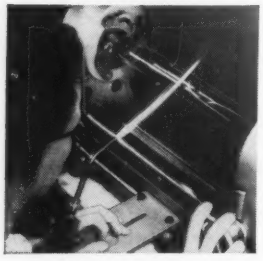

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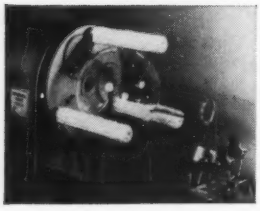

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Continued from page 64a

To register in advance, mail the registration card with the applicable fee (regular fee—$15.00, student fee—$10.00) to the Federation Convention Office, 9650 Wisconsin Avenue, Washington 14, D. C. *Advance registration will be accepted until March 31.* A validated receipt will be mailed to you. BRING THIS RECEIPT WITH YOU TO THE ANNUAL MEETING. Upon presentation of this receipt at the Advance Registration desk, you will receive your official badge which *must* be worn for admission to scientific sessions, exhibits, and the Placement Service.

Guest Registration, with a fee of $2.00, is available to non-scientist members of the families of registrants, including women who wish to join the Ladies' Program.

Scientists who register in Atlantic City on Thursday, April 19, the last day of scientific sessions, will be admitted at a reduced fee of $8.00.

PROGRAM

A new pocket-size Program will be mailed prior to the Meeting to each member of the Federated Societies and to each advance registrant. It will also be given to each non-member registering at Atlantic City. Additional copies of the Program and FEDERATION PROCEEDINGS, March 1962 (Abstracts), will be on sale in the registration area.

TRANSPORTATION FACILITIES

The Federation and the Atlantic City Convention Bureau will again join efforts to facilitate travel into and out of Atlantic City. During the Annual Meeting, shuttle buses will travel between the Philadelphia Airport and Atlantic City on a regular schedule. The Federation Convention Office will mail the bus schedule to all Federation members, and to others on request, early in February. To ensure good connections, refer to the bus schedule and *make your reservations early.*

HOTEL RESERVATIONS

A Housing Bureau will serve members and non-members attending the Federation Meeting. The official form for hotel reservations must be used to obtain special Federation hotel rates in Atlantic City. These forms will be mailed on request from the Federation Convention Office. Please address hotel forms to: 1962 Federation Meeting, Housing Bureau, 16 Central Pier, Atlantic City, New Jersey. Confirmations will be forwarded directly from the hotels. The Atlantic City hotels have assured us that rooms will be delivered at the price confirmed.

DEADLINE FOR ABSTRACTS

December 30, 1961, is the deadline for the receipt of abstracts by the Society Secretaries. Abstracts received on or after December 31, 1961, will not be accepted.

EXHIBITS

Industrial and institutional exhibits will be located on the Main Arena Floor, Convention Hall. They will be open from 8:45 a.m.–5 p.m., Saturday, April 14, through Wednesday, April 18; and from 8:45 a.m.–12 noon on Thursday, April 19. Member exhibits are no longer accepted for Federation Annual Meetings.

For information and application forms for institutional exhibits, write to the Federation Convention Office. January 10, 1962, is the deadline date for receipt of completed application forms.

PLACEMENT SERVICE

The PLACEMENT SERVICE will be located in the Lower Lobby of Convention Hall. To use this facility, *both* employers and candidates must have paid the registration fee for the Annual Meeting and also be registered with the Placement Service.

An employer wishing to arrange for *personal interviews* with candidates must be either an annual subscriber to the Placement Service Lists or subscribe to the February 1962 List of Candidates. TO AVOID DELAY at the Employers' Registration desk each employer should make certain in advance that, if a subscription is not in his own name, he has received the proper authorization from the subscriber to use the *personal interview facilities*, i.e., if the subscription is entered from a Department, Library or Purchasing Office, a form authorizing *not more than three* named representatives to use the facilities must have been filed with the Placement Service Office before March 30.

A list of available positions will be published in March, the final list before the Annual Meeting. Completed registration cards for positions to be included in the March List must be submitted to the Washington Office before February 26. Facilities will be provided at the meeting for employers to list additional positions.

Candidates who wish to be included in the February 1962 List of Candidates must file their applications before February 1. Advance registration for candidates who wish to use the Placement Service but do not wish to be listed in the February bulletin will be accepted until March 30.

Employers and candidates may register at Atlantic City, but the procedure will be more time-consuming.

All correspondence regarding this activity should be directed to:

FEDERATION PLACEMENT SERVICE
9650 Wisconsin Avenue
Washington 14, D. C.

SPECIAL FUNCTIONS

The Federation Convention Office will assist with arrangements for meal functions and meetings of informal groups. For reservation forms and information, write to the Federation Convention Office. If time and location of these events are to be listed in the Program, all arrangements must be completed by February 2, 1962.

Tickets to special functions will be on sale in the registration area, Lobby, Convention Hall.

WOMEN'S HOSPITALITY LOUNGE

A Women's Hospitality Lounge will be located in the Founders Room of the Dennis Hotel. Upon payment of a registration fee of $2.00, wives and other non-scientist family members of registrants are invited to use the Lounge as a meeting place and social center from 9 a.m. to 5 p.m., Saturday, April 14, through Wednesday, April 18. Free coffee service, card tables and cards will be provided.

A special program for ladies has been arranged for Tuesday, April 17. Buses will leave the Dennis Hotel for historic Smithville Inn where time will be allowed for browsing through the Colonial shops, followed by luncheon and a hat show. On the return trip a visit will be made to a nearby winery for a tour and free refreshments. Tickets for this excursion will be on sale in the Hospitality Lounge, and at the Ticket Sales desk in the Lobby, Convention Hall.

Women may register in the Hospitality Lounge from 9 a.m. to 5 p.m. on Saturday and Sunday, April 14 and 15. They may also register at the Guest Registration desk in the Lobby, Convention Hall, during all regularly scheduled registration hours, starting at noon on Friday, April 13.

Continued on page 78a

FORMS AND ANNOUNCEMENTS

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THE 1962 ANNUAL MEETING

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Joint Session of the Federation

APPROACHES TO STUDY OF DISEASE[1]

New approaches in the study of cardiovascular disease: aldosterone, renin, hypertension and juxtaglomerular cells[2]

W. STANLEY HARTROFT AND PHYLLIS M. HARTROFT[3]

Department of Pathology, Washington University Medical School, St. Louis, Missouri

IN A RECENTLY PUBLISHED EDITORIAL in the *British Medical Journal* (38), entitled, "Regulation of Aldosterone Secretion", it was stated: "The juxtaglomerular apparatus, which increases in size during a recognized stimulus to aldosterone secretion, ... is an obvious candidate for the honour" (of regulating its secretion). Since the initial discovery of juxtaglomerular cells (in the kidneys of mice (39)), considerable evidence for this candidacy has accumulated, especially during the past decade. The purpose here is to present the available evidence supporting the concept that renin is secreted by juxtaglomerular cells, and that it acts as a trophic hormone on zona glomerulosa of the adrenal cortex thereby regulating the production and liberation of aldosterone.

We have chosen this subject for not only its own interest, but also because it exemplifies the approach of the experimental pathologist, utilizing abnormal material from both animals and man. The evidence to be cited will demonstrate how advances in the laboratory using animals, have driven us to the autopsy table for their confirmation and extension in human material; and they, in turn, have indicated further animal experiments. This type of approach seems to us the unique essence of experimental pathology, distinguishing it from experimental biology in general.

HISTOLOGY, HISTOCHEMISTRY AND ULTRASTRUCTURE

Although first described more than a third of a century ago, the morphologic relations of juxtaglomerular cells are not widely appreciated. They occupy the wall of the afferent glomerular arteriole, replacing the smooth muscle cells of the media at this point (Fig. 1). Of particular interest is their relationship to the macula densa of the distal portion of the tubule. It is this portion of the nephron that returns to that glomerulus from which it arises to occupy a position at the glomerular pole. Macula densa and juxtaglomerular cells of the same nephron are therefore directly in contact at this site. As emphasized by McManus (32), the basement membranes here between arteriole and tubule become extremely attenuated and even absent. Under certain abnormal conditions, increases in size and number of juxtaglomerular cells are most prominent in the portion of the arteriolar wall closest to the macula densa, particularly in dog and man (28).

The granules which occupy cytoplasm of juxtaglomerular cells vary greatly under normal conditions from species to species. They are exceptionally prominent and numerous in the mouse, the species in which the cells were first discovered. In man, although present, they are few and inconspicuous, forming incomplete, poorly visualized perinuclear haloes. They can be stained in a variety of ways, appearing slate gray-blue by the trichrome techniques which had been used originally to demonstrate them. This stain differentiates them poorly from the surrounding rodlets of the tubule-cytoplasm. A modification of an islet cell stain (Bowie) colors the granules selectively, such an intense purple that they stand out even under low magnifications of the light microscope (Fig. 2). It is possible, using this stain, to count rapidly groups of juxtaglomerular cells in relation to the number of glomeruli present in the same sections. By weighting these counts according to the intensity of granulation, an Index of Juxtaglomerular Granulation (JGI) can be formulated[4] which gives

[1] April 11, 1961, Atlantic City, N. J.

[2] This work has been generously supported for over a decade by the Nutrition Foundation, to which we express our thanks; together with the Public Health Service (Grant No. A-1114).

[3] Present address: The Research Institute, Hospital for Sick Children, 555 University Avenue, Toronto 2, Ontario, Canada.

[4] $\text{JGI} = \frac{\text{No. of weighted JG-groups}}{\text{No. of glomeruli}} \times 100$. Weighting is done by multiplying by 1, 2 or 4 according to degree of granulation.

reproducible results with a reasonably small standard error (25) even when carried out by different observers. The more intense the granulation and the greater the numbers of juxtaglomerular cells, the higher this JGI (and presumably the greater the manufacture and liberation of their secretory product).

Histochemical studies have revealed that the granules stain intensely by McManus' periodic acid Schiff technique (33) and may be composed of a ketosteroid

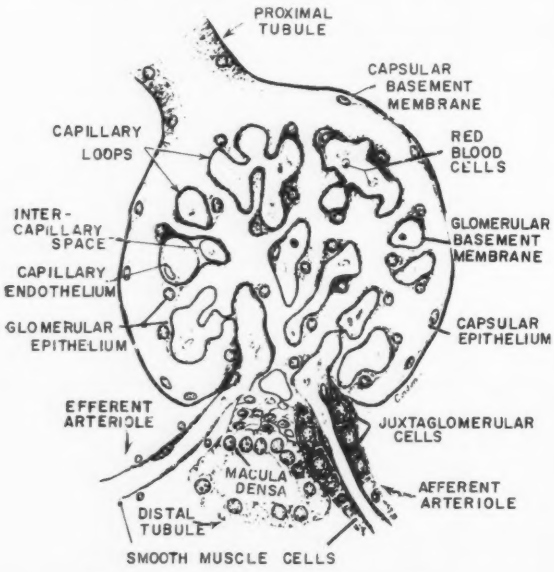


FIG. 1. Drawing of a glomerulus showing position of juxtaglomerular cells in the wall of the afferent arteriole. (From Ham, A. W., *Histology*, 2nd ed., Philadelphia: Lippincott, 1953, p. 602.)

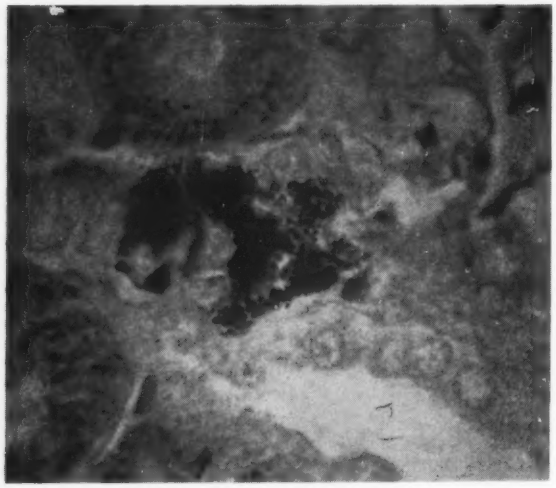


FIG. 2. Juxtaglomerular cells of a rat in a section stained by the Bowie technique, granules (black in the photo) contrast sharply with the background. Glomerulus lies to the upper right; macula densa to the lower right (×1500). (From Ham, A. W., *Histology*, 2nd ed., Philadelphia: Lippincott, 1953, p. 604.)

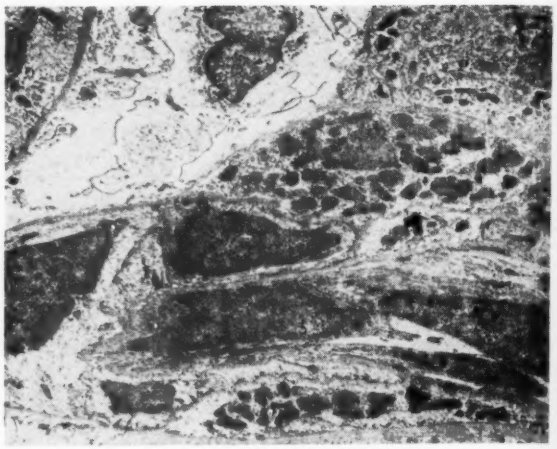


FIG. 3. Osmophilic granules in a juxtaglomerular cell can be seen (upper middle right) in this low power electron micrograph of an afferent arteriole in the kidney of a rat. A portion of a macula densa cell is seen in contact with a juxtaglomerular cell at the upper right (×5000). (From Hartroft, P. M. and L. N. Newmark, *Anat. Record* 139: 185, 1961.)

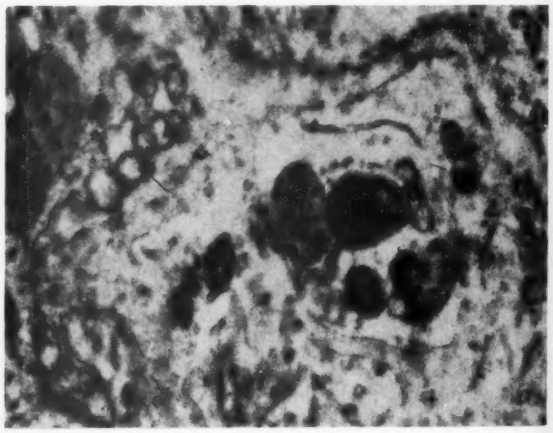


FIG. 4. A group of four granules can be seen at the middle right of this electron micrograph of juxtaglomerular cells in the kidney of a sodium-deficient dog. Note "granules within granules" (×20,000). (From Hartroft, P. M. and L. N. Newmark, *Anat. Record*, 139: 185, 1961.)

protein complex (21). They are osmophilic, permitting their identification as dense bodies in electron micrographs (Fig. 3 and 4). Electron microscopy demonstrates that the morphology of the granules is characteristic of true, specific secretory bodies analagous to those in other endocrine organs such as beta cells of the Islets of Langerhans or others in the anterior pituitary (27). The granules are larger, however, than those of either the islets or the pituitary and are almost equal in size to zymogen granules of pancreatic acinar cells. High magnifications achieved with the electron microscope suggest an internal structure within some of the larger juxtaglomerular cell-granules (Fig. 4). These

observations definitively contradict the assumption made by some that the granules are merely degenerative hyaline-like bodies. Their true secretory nature is established by electron micrographs.

JUXTAGLOMERULAR CELLS AND SODIUM

Goormaghtigh in Belgium studied juxtaglomerular cells in human autopsy material and observed increases in their granulation and size in hypoxic renal necrosis (15) and malignant hypertension and other conditions (14). But it was not until 1949 that Dunihue (10) reported the first consistent alterations in these cells to be produced at will in animals. He found that in bilaterally adrenalectomized cats, the renal juxtaglomerular cells enlarged, increased in number and became abnormally hypergranulated. He attributed this change to the fall in blood pressure induced by adrenalectomy. But the thought occurred to us that this change might be associated just as well with sodium loss in the urine of the animals. To test this hypothesis, groups of intact rats were fed various levels of dietary sodium (low, adequate, and excess) with and without administration of desoxycorticosterone acetate (DCA) (24).

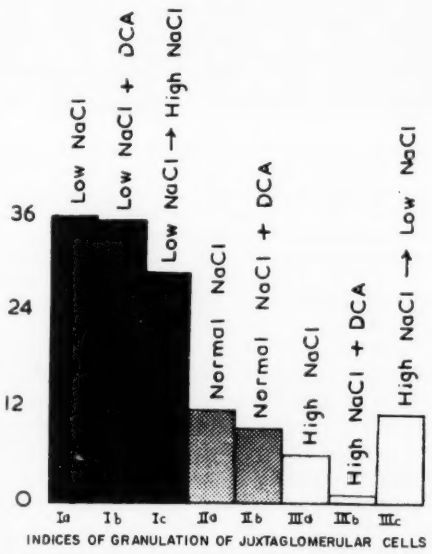


FIG. 5. Changes in granulation of juxtaglomerular cells in young adult rats fed varying amounts of sodium for 2–4 weeks are shown graphically. Each bar represents 9–10 rats. Rats in groups Ic and IIIc were transferred to high and low sodium intakes, respectively, for the last few days of the experiment. (From Hartroft, P. M. and W. S. Hartroft, *J. Exptl. Med.* 97: 415, 1953.)

The results, summarized in Fig. 5, supported the idea that loss of sodium affects juxtaglomerular granulation in the absence of any significant alteration in the level of blood pressure. Rats fed a low sodium diet exhibited very significant increases in numbers and granulation of their juxtaglomerular cells (high JGI) and exogenous DCA failed to prevent this change. DCA, however, did lower JGI significantly, although not strikingly, in rats fed a normal amount of dietary sodium; and, when given rats fed excess sodium, which by itself lowered JGI, DCA almost abolished granulation (JGI almost zero). This experiment was the first of many to indicate the close relation of sodium metabolism and excretion to juxtaglomerular cells. These studies were later repeated in cats and effects of dietary sodium restriction in this species on juxtaglomerular cells compared directly with effects of adrenalectomy as previously reported by Dunihue. The results (Fig. 6) indicate a highly statistically significant increase in JGI corresponding to either duration of dietary sodium deficiency or length of postoperative interval after adrenalectomy (35). An inverse correlation (Fig. 7) exists in both groups of experimental animals between JGI and levels of serum sodium.

An example of the striking increase in size, number and granulation of juxtaglomerular cells of a sodium-deficient cat is shown in Fig. 8. Increase of JGI induced

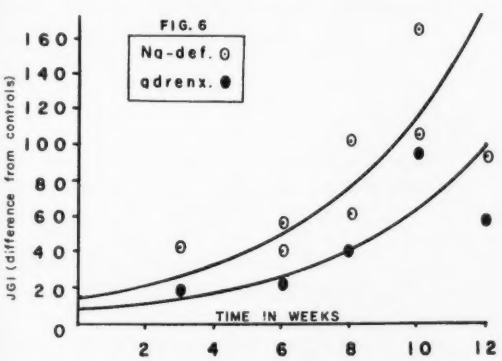


FIG. 6. Increase in granulation of juxtaglomerular cells of cats over a period of 12 weeks is shown. Each point represents a difference from a littermate control cat. The upper regression curve represents sodium-deficient cats (open circles) and the lower curve represents adrenalectomized cats (solid circles) maintained with cortisone on a normal sodium intake.

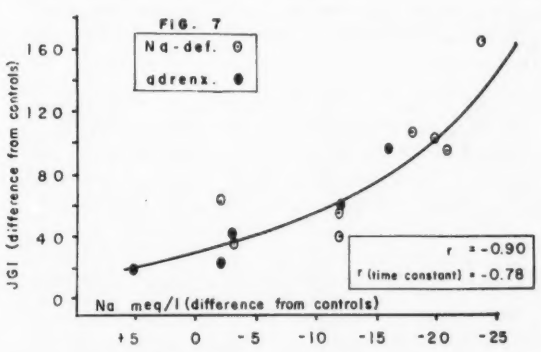


FIG. 7. Correlation between granulation of juxtaglomerular cells (JGI) and serum sodium in sodium-deficient (open circles) and adrenalectomized (solid circles) cats is statistically significant. Same animals are represented in Fig. 6. The correlation coefficient with or without time held constant is highly significant. With a decrease in plasma sodium, there is an increase in JGI.

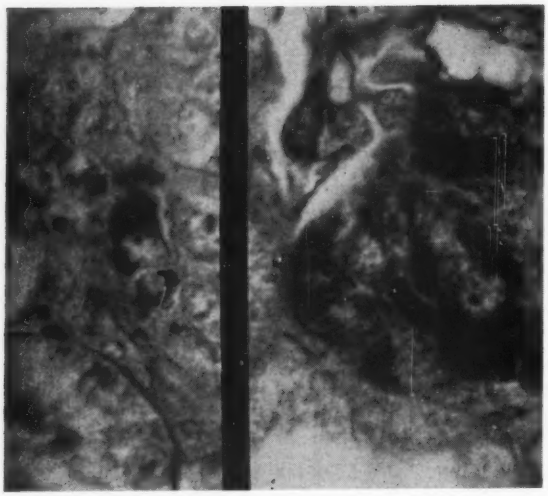


FIG. 8. Juxtaglomerular cells in cats. On the left is a typical field from a control cat, showing dark juxtaglomerular cell granules. On the right is a representative field from a sodium-deficient cat showing hypergranulation and hyperplasia of juxtaglomerular cells. In both, the glomerulus is just out of the field above. Bowie stain (×1000).

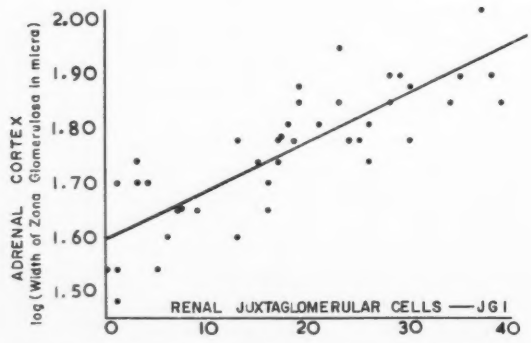


FIG. 9. A high correlation exists between width of the zona glomerulosa of adrenal cortex and degree of granulation of juxtaglomerular cells (JGI) in rats fed varying amounts of sodium. (From Hartroft, P. M. and W. S. Hartroft, *J. Exptl. Med.* 102: 205, 1955.)

by lowering serum sodium by dietary or other means has also been demonstrated in dogs, rabbits and man, as shown below. Even when sodium deficiency has not advanced to the stage of actual lowering of levels of sodium in serum, increases in juxtaglomerular granulation can be demonstrated. This observation suggests that juxtaglomerular cells are actively involved in homeostatic regulation of sodium under abnormal conditions.

JUXTAGLOMERULAR CELLS AND ZONA GLOMERULOSA OF ADRENAL CORTEX

In the studies described above, it soon became apparent that a certain degree of variation in the response of juxtaglomerular cells to dietary sodium deficiency

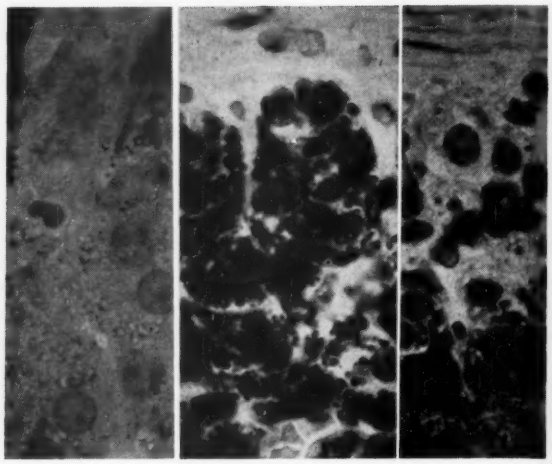


FIG. 10. Frozen sections stained with Oil Red O of zona glomerulosa of adrenal cortex from rats are illustrated. A control is shown at the center (capsule above, limit of the zone at bottom, lipid gray). On the right is a rat fed excess sodium showing atrophy and lipid depletion of zona glomerulosa. On the left, hypertrophy and lipid depletion in zona glomerulosa of a sodium-deficient rat. Only a portion of the zone could be included at this magnification (×1000). (From Hartroft, P M. and W. S. Hartroft, *J. Exptl. Med.* 102: 205, 1955.)

existed from animal to animal. This observation prompted a search for other changes, in addition to hypergranulation of juxtaglomerular cells, that might correlate in some manner.

Attention was soon directed to the zona glomerulosa of the adrenal cortex because of previously established evidence linking this zone with sodium metabolism (8). A standard method was developed for measuring the width of the zone in fat-stained frozen sections of uniform thickness (5μ) of adrenals cut in equatorial planes. When logarithmic values of these measurements were plotted against JGI's of normal and sodium-deficient rats, a straight-line correlation of high significance was established (26) (Fig. 9). Examples of the histologic appearance of zonae glomerulosae from rats fed various levels of dietary sodium are illustrated in Fig. 10. It was apparent at this point, that a three-way correlation existed between levels of serum sodium, intensity of granulation of juxtaglomerular cells and width of zona glomerulosa. Further studies in collaboration with Dr. A. Eisenstein then established that width of zona glomerulosa in similar animals correlated with amounts of aldosterone produced by such adrenals incubated in vitro (12, 23). It now seemed important to attempt to obtain relevant data from human material.

JUXTAGLOMERULAR CELLS, ZONA GLOMERULOSA AND SERUM SODIUM IN MAN

These studies, carried out with Dr. James Pitcock, utilized human autopsy material obtained from patients of Barnes Hospital. JGI's were estimated for patients from whom at least three determinations of their levels

of serum sodium were available during the last week prior to death. When the average of these three readings was plotted against logarithmetic values of the corresponding JGI's, a straight line correlation (inverse) with a coefficient of 0.61 ($P < 0.01$) (Fig. 11) was obtained (36). In these same patients width of zona glomerulosa of the adrenal was measured by a standard method and also plotted against logarithms of the same patient's JGI. Again a straight line correlation (this time, direct of course) was obtained with a coefficient of 0.49 ($P < 0.05$). Examples of hyperplasia of adrenal cortex (Fig. 12) and hypergranulation of juxtaglomerular cells in man (Fig. 13) are illustrated, the last from a case of Addison's disease, as first noted in this condition by McManus (34). It was not possible in the case of this human material, as it had been for the rats, to establish a correlation between width of zona glomerulosa and amount of aldosterone production by their adrenals incubated in vitro.

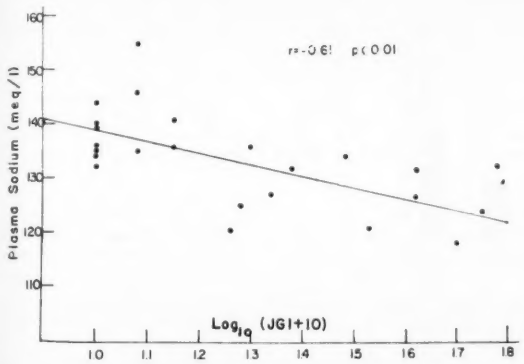


FIG. 11. Correlation of plasma sodium and degree of granulation of juxtaglomerular cells in man is highly significant. (From Pitcock, J. A. and P. M. Hartroft, *Am. J. Pathol.* 34: 863, 1958.)

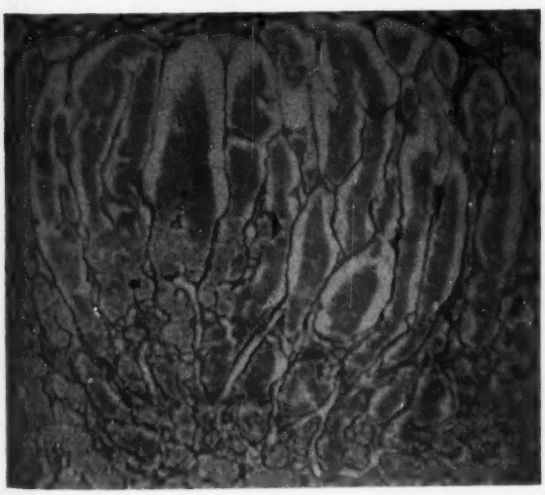


FIG. 12. Nodular hyperplasia of zona glomerulosa of adrenal cortex from a patient with hyponatremia. Reticulin stain (×150). (From Pitcock, J. A. and P. M. Hartroft, Am. J. Pathol. 34: 863, 1958.)

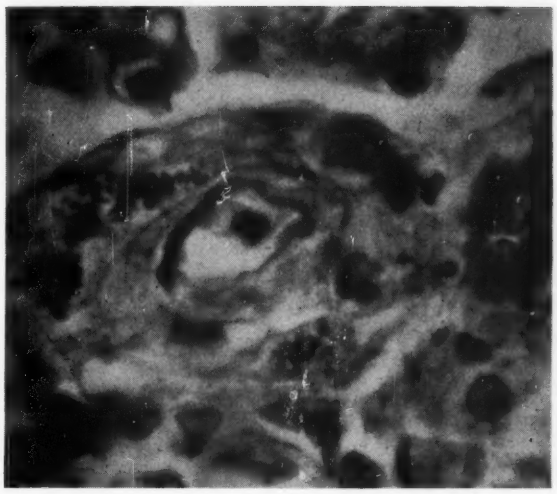
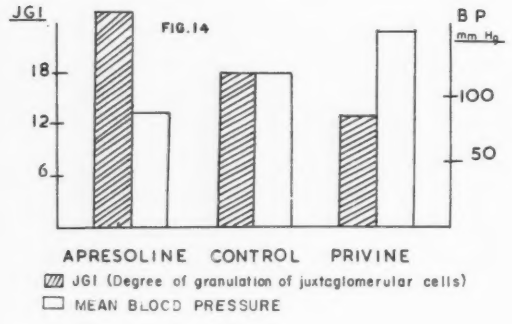


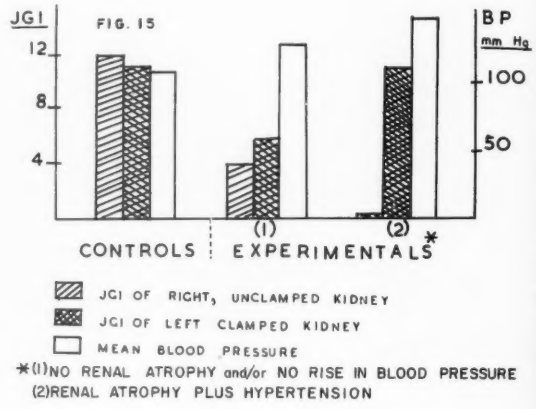
FIG. 13. This afferent arteriole has been cut in cross section in a kidney from a case of Addison's disease and shows hyperplasia of juxtaglomerular cells. These cells in man are normally sparsely granulated in contrast to the increase in size and numbers of granules seen here. Bowie stain (×1000).

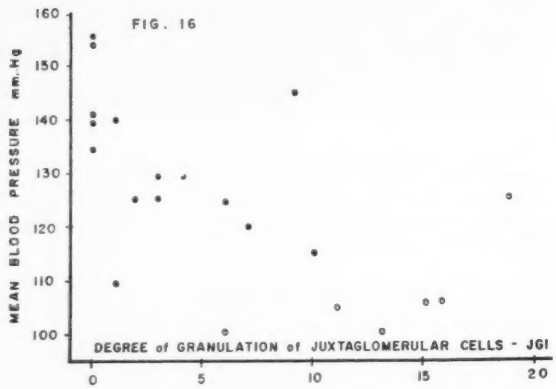
JUXTAGLOMERULAR CELLS AND EITHER TRANSIENT OR SUSTAINED HYPERTENSION

Previously established relations between kidneys, adrenals, DCA or aldosterone, and hypertension indicated the need for studies of juxtaglomerular cells in hypertensive states. Goormaghtigh (loc. cit.), Dunihue (9) and others suggested that juxtaglomerular cells might play a role in regulation of blood pressure. Initial studies were carried out by producing temporary changes in blood pressure (over periods of several weeks) by extrarenal pharmacologic means (repeated injections of a vasodepressor (Apresoline) or a vasoconstrictor (Privine) in rats). In these animals lowering of blood pressure increased the JGI; elevation, a fall (Fig. 14) (26). The extrarenal etiology of the changes in blood pressure was deemed an important feature in these preliminary experiments in order to establish that changes in levels of blood pressure could alter the appearance of juxtaglomerular cells in the absence of direct renal manipulation. Having obtained positive results, juxtaglomerular cells in experimental renal hypertension were then studied.

Sustained elevations of blood pressure were produced in rats by partial constriction of one renal artery. The animals comprised three groups; sham-operated controls, those with renal artery constriction that developed moderate degrees of hypertension, and those with constricted arteries that developed severe hypertension (Fig. 15) (22). The more severe the hypertension, the lower the JGI of the "unprotected" or unclamped kidney, whereas the JGI of the ischemic kidney did not







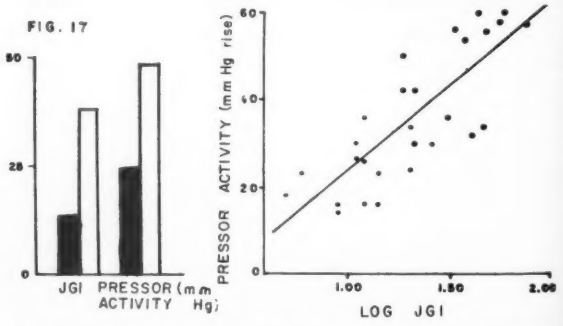


FIG. 14. Effects of temporary changes in blood pressure on juxtaglomerular cells. Level of blood pressure and degree of granulation of juxtaglomerular cells (JGI) in rats injected with vasoactive substances for 3 weeks. The lower the blood pressure, the higher the JGI.

FIG. 15. Effect of unilateral partial constriction of the renal artery on juxtaglomerular cells. Experimental hypertension in rats. In rats with severe degree of hypertension (2), degree of granulation of juxtaglomerular cells in the contralateral "untouched" kidney was almost zero.

FIG. 16. Inverse correlation of the level of blood pressure and of degree of granulation of juxtaglomerular cells in the contralateral "untouched" kidney of rats with the opposite renal artery partially constricted ($r = -0.7$). (From Hartroft, P. M., *J. Exptl. Med.* 105: 501, 1957).

FIG. 17. Degree of granulation of juxtaglomerular cells and pressor activity (renin) of the same kidneys from sodium-deficient (open bars) and control (solid bars) adult rats. On the left it is seen that both parameters were increased in sodium-deficient animals. On the right, JGI plotted against pressor activity yielded a highly significant correlation coefficient (0.08). (Modified from Pitcock, J. A., P. M. Hartroft, and L. N. Newmark, *Proc. Soc. Exptl. Biol. Med.* 100: 868, 1959.)

alter in a significant fashion, although a tendency for an increase in granulation was noted in some instances.

When the degree of granulation in the juxtaglomerular cells of the contralateral kidney was plotted against the level of blood pressure (Fig. 16), the inverse relationship was clearly apparent. The cytoplasm of degranulated cells of the contralateral (unclamped) kidney was notably pale and clear. A similar observation has since been reported in the juxtaglomerular cells of nonischemic kidneys in cases of unilateral renal hypertension in man (41). We have suggested that a convenient term for this condition might be "clear cell hypertension."

JUXTAGLOMERULAR CELLS AND RENIN

The evidence up to this point rather strongly pointed to the possibility that the product of juxtaglomerular cells (represented in stored intracellular form by their secretory granules) might act as a trophic hormone on the zona glomerulosa of the adrenal cortex to stimulate production and liberation of aldosterone in states of sodium depletion. The evidence in the available literature suggested that this substance derived from juxtaglomerular cells might be renin, because (Table 1) changes in amounts of extractable renin in the kidney under a variety of conditions paralleled changes in the same direction in the JGI under similar conditions as reported above. This evidence however was of highly indirect

TABLE 1. *Indirect Comparisons of Juxtaglomerular Cell Granulation and Renin*

	JG Cell Granules	Renin
DCA and excess salt	↓ (24)	↓ (18, 19)
Contralateral kidney in unilateral renal ischemia	↓ (22)	↓ (17)
Adrenalectomy	↑ (10)	↑ (3, 20)
Renal ischemia	↑ (16)	↑ (13)

Figures in brackets refer to references.

nature, the data having been obtained in different laboratories and from completely different animals. Based on this line of evidence, experiments were therefore designed to test the hypothesis concerning the source of renin and its action on the adrenal as directly as possible.

Bio-assays were carried out to measure pressor activities (renin) of extracts of kidneys of sodium-deficient rats (37), in hypertensive rats (40) and in rats fed excess

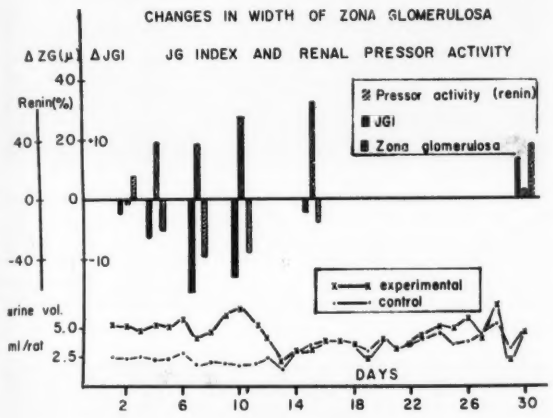


FIG. 18. Graph showing changes in rats injected daily with renal extracts from dogs with hypergranulated juxtaglomerular cells and excess renin content. The O line represents control rats injected with the same dilution of renal extracts from kidneys of control dogs with sparsely granulated juxtaglomerular cells. Changes occur during the first 10 days after which rats develop antibodies to the injected extracts (determined by bioassay). ZG: zona glomerulosa; JGI: degree of granulation of juxtaglomerular cells.

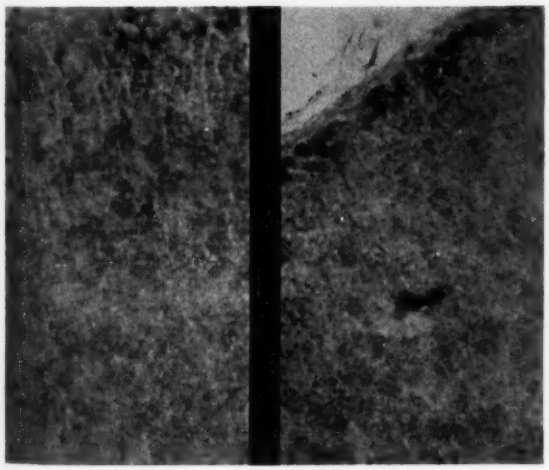


FIG. 19. Frozen sections of adrenal cortex stained with Oil Red O from rats injected with renal extracts for 1 week. On the right, a control rat injected with renal extract from a control dog. Zona glomerulosa is normal. On the left, a rat injected with renal extract from sodium-deficient dogs with hypergranulated juxtaglomerular cells. Zona glomerulosa is hypertrophied and partially depleted of lipid (×100).

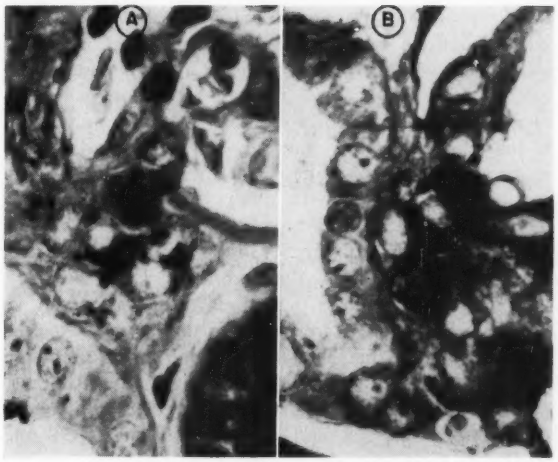


FIG. 20. Juxtaglomerular cells of dogs stained by the Bowie technique. On the left, a control dog with only a few granules in juxtaglomerular cells — normal for this species. On the right, hyperplasia and hypergranulation of juxtaglomerular cells in a dog with secondary hyperaldosteronism produced by constriction of the thoracic vena cava (×1200). (Experiment in collaboration with James O. Davis and co-workers, National Heart Institute (2)).

sodium (29). JGI's were estimated for the same kidneys. The results are contrasted on the left in Fig. 17 for sodium-deficient and control rats, and on the right the correlation between pressor activity and JGI in each case is plotted.

Another approach to the problem consisted of injecting normal rats with renal extracts prepared from sodium deficient dogs (high JGI; high pressor activity of extract) and contrasting these animals with others treated with similar extracts prepared from the kidneys of normal dogs (relatively low JGI; relatively low pressor activity of extract). The width of zona glomerulosa in the rats receiving renal extracts prepared from the sodium-deficient dogs was almost twice that in rats receiving renal extracts from the normals. Mitotic activity was similarly increased. The JGI of rats receiving the pressor-rich extracts was significantly lower than that for controls receiving extracts of kidneys of normal dogs (Fig. 18) (30). If renin is the produce of juxtaglomerular cells, we would expect that extracts rich in exogenous renin would diminish granulation representing endogenous renin, as observed. If renin is capable of stimulating zona glomerulosa (and aldosterone production), exogenously administered renin (in the pressor-rich extracts of the sodium-deficient dogs' kidneys) should widen the zona glomerulosa of the recipient rats as occurred. The evidence that widening of zona glomerulosa in rats is associated with increases in aldosterone production in vitro had already been obtained (loc. cit.). Examples of widening of the zona glomerulosa produced by renin-rich extracts administered to weanling rats are shown in Fig. 19.

Davis et al. (7) had established by direct measurements

that levels of aldosterone are increased above normal in blood obtained from adrenal veins of dogs in which ascites followed narrowing of the thoracic portion of the inferior vena cava. These animals represent an experimental form of secondary aldosteronism comparable to that encountered in man. Dr. Davis was kind enough to provide us with kidneys obtained from a series of such animals, along with others from their controls. The degree of granulation of their juxtaglomerular cells (JGI) was significantly higher in the dogs with secondary aldosteronism than in their controls (Fig. 20) (2). Recourse was then had to human autopsy material, in which JGI and width of zona glomerulosa were measured in patients suffering secondary aldosteronism associated with cardiac failure or cirrhosis with ascites. In these patients, compared with suitably selected controls of comparable age, sex and race, the average JGI and width of zona glomerulosa were significantly greater (unpublished data). The autopsy data, therefore, appear to support the results of the animal experiments, indicating hyperactivity of juxtaglomerular cells in secondary aldosteronism.

The essential role of the kidney in regulation of aldosterone secretion has recently been demonstrated by Davis et al. (1, 6), who carried out a series of experiments involving hypophysectomized bilaterally nephrectomized dogs. Furthermore, their work, as well as those of others, (4) indicates that renin (or angiotensin) may be the specific aldosterone-stimulating hormone.

FLUORESCENT ANTIBODY TECHNIQUE APPLIED TO JUXTAGLOMERULAR CELLS

When pure antigen is available, the fluorescent antibody technique of Coons and Kaplan (5) is capable of providing rather direct evidence to indicate sites of hormone production, as has been established for insulin,

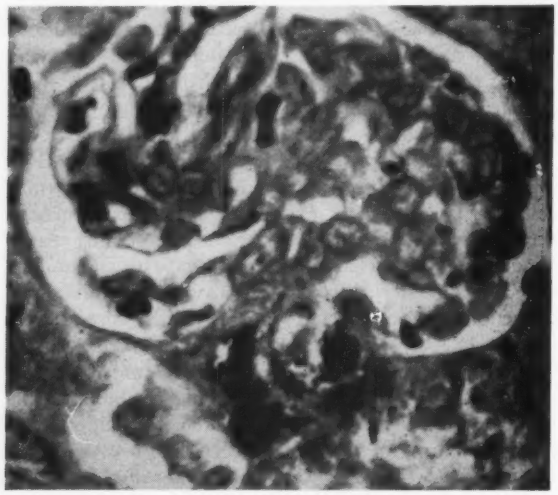


FIG. 21. Bowie stain of juxtaglomerular cells of a sodium-deficient rabbit (3½ weeks) for comparison with Fig. 22.

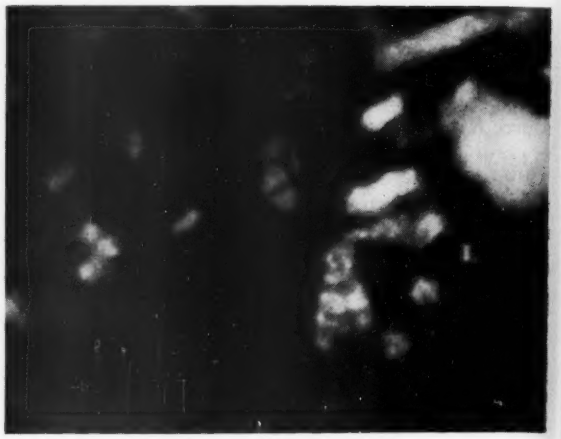


FIG. 22. Frozen-dried section of kidney from same rabbit as in Fig. 21. Section was stained with fluorescein-labeled antirenin. Note specific staining of granules in juxtaglomerular cells (lower right). Tubule at upper right shows autofluorescence (blue-white in contrast to yellow-green of juxtaglomerular cells). (From Edelman, R. and P. M. Hartroft, *Circulation Research* accepted for publication, 1961.)

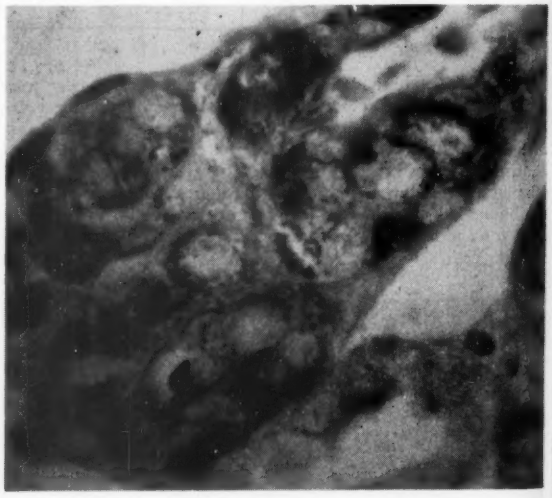


FIG. 23. Tangential section of afferent arteriole from a weanling rabbit fed a sodium-deficient diet for 8 weeks. Note extreme hypergranulation and hyperplasia of juxtaglomerular cells. For comparison with Fig. 24. Bowie stain.

for example (31). Even the most highly purified preparations of renin as obtained by Goldblatt are, however, far from free of impurities. Although pure preparations of renin are unavailable at the present time, and although fully aware of the possibility that the impurities might introduce misleading results, the fluorescent antibody technique was undertaken to determine if antirenin labeled with fluorescein isocyanate, would localize in juxtaglomerular cells (11).

Dogs were treated with repeated injections of Freund's adjuvants and partially purified preparations of renin

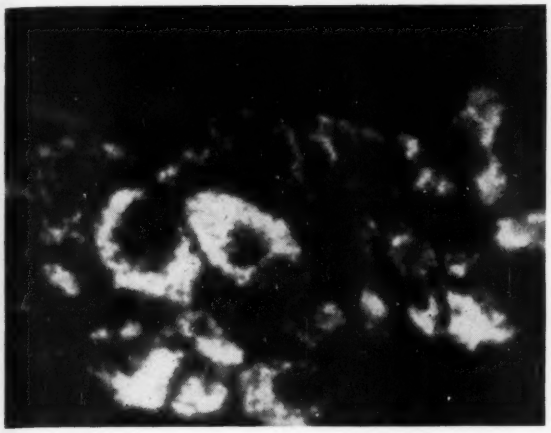


FIG. 24. Frozen-dried section of kidney from same animal as in Fig. 23, stained with fluorescein-labeled antirenin. Note bright specific fluorescence of individual juxtaglomerular cell granules.

kindly supplied by Dr. Harry Goldblatt and Dr. E. Haas, and blood collected when the animals' titers of antirenin, as measured by bio-assay, were at their height. Serum was adsorbed with liver powder and the antibodies conjugated with fluorescent dye. Frozen-dried sections of kidney from normal and sodium-deficient rabbits were incubated at room temperature for 15–30 min with the labeled antibody, rinsed in two changes of buffered saline, mounted in glycerin and examined under the ultraviolet microscope. Adjacent sections were cut and stained by the modification of Bowie's method (36) for comparison.

Two examples of comparable fields containing juxtaglomerular cells in sections stained by the fluorescent antibody technique and by the modified Bowie method are illustrated in the last four figures (Fig. 21 to 24). Specific fluorescence (representing the antibody-an igen linkage) of not merely juxtaglomerular cells, but of their individual granules resulted. The reaction could be blocked by incubating the labeled antibody solutions with extracts rich in renin prior to treatment of the sections and could be significantly diminished by first incubating the sections with unlabeled antibody. Although this evidence for the juxtaglomerular origin of renin is admittedly presumptive, because of the impure nature of the renin used for antibody production, when taken together with the data presented in the foregoing section, the hypothesis seems to stand on relatively firm ground.

CONCLUSIONS

The evidence summarized in this paper, we believe, strongly indicates that production of aldosterone by zona glomerulosa of the adrenal cortex is regulated by renin acting as a trophic hormone on the adrenal. Indirect evidence, derived not only from the available literature, but, also, from data obtained from the experiments described here, indicates that the most likely source of renin is the juxtaglomerular cell. The anatomic position of these cells places them in an exceptionally advantageous position for regulation of aldosterone production (blood pressure and sodium retention) because of their interposition between the macula densa of the distal tubule and the lumen of the afferent arteriole carrying blood to the glomerulus. But the mechanism by which these cells take advantage of this unique situation for aldosterone regulation remains to be elucidated. It constitutes yet another challenge to experimental pathologists in their approach to the study of related diseases, including hypertension.

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Histochemistry and electron microscopy in the study of disease of the kidney

J. F. A. McMANUS

Department of Pathology, University of Alabama Medical Center, Birmingham, Alabama

THE MATERIAL WHICH I AM GOING TO REVIEW with you is largely obtained from cases of human disease, in which we are studying the "experiments of nature." We lay claim to the "experimental" part of our Federation's title and we claim to be biologists, inasmuch as we are pathologists. A pathologist by definition is a student of disease. When we examine the meaning of the word "disease," we find that it can best be taken to include everything that decreases life in its powers, duration, or enjoyment. There can be no disease without life; and the pathologist, as a student of disease, shares the fundamental interest in living things that characterizes all biologists.

The methods and aims of the pathologist are those of the biologist. In studying the disease conditions which I am going to review with you, my colleagues and I have used the clinical histories, which you might call the study of the ecology, as well as the methods of anatomy, biochemistry and, where possible, those of physiology in the tests performed on the clinical patients.

As the title of this presentation suggests, we are going to speak only of histochemistry and electron microscopy. Even in those fields, the amount of data that have been collected is so very great that I am forced to limit my discussion to areas in which my colleagues and I at the University of Alabama Medical Center, and for three years at the University of Virginia, have been particularly engaged. These studies have been generously supported by the National Institutes of Health and the Department of Health, Education and Welfare.

I will review with you instances in which the marks of disease can be recognized only by histochemistry, and instances in which histochemistry does not show changes, while electron microscopy does. Further, I will review with you something of the natural history of two disease conditions in the kidney. Finally, we will study this material and attempt to define something of the limits and capacity of the techniques described.

I show you first, for purposes of orientation, the appearance of a normal human renal glomerulus stained by the periodic acid schiff or PAS stain. Observe the basement membrane coloring magenta, or purple, smoothly covering the capillary loops, except where several loops lie together. I do not propose to raise the problem of glomerular structure, but will pass directly to the study of the next slide, which is one from a case of Toxemia and pregnancy. The smooth capillary basement membrane of the normal glomerulus is replaced by one which is vacuolated and duplicated, and this vacuolation is most conspicuous where the capillaries come together, the site of the interluminal cell aggregates (1).

This change was very largely overlooked before the introduction of the PAS histochemical method. The PAS stain allowed the identification of the specific renal glomerular change of toxemia of pregnancy. Identification and diagnosis of a lesion is a taxonomic problem, akin to the precise placing of the animal, say a beetle, or a plant or leaf, in its proper biological category.

The next slide is that of tubulo-venous thrombosis (3). The tubule containing a cast has eroded into an adjacent vein and thrombosis has been laid down at the site. This lesion is typical and diagnostic of renal failure following severe crushing injuries of the limbs and body or following shock episodes in general. Found three days following the precipitating injury or accident, it allows us to say that it is probably a case of so-called "crush kidney," but it does not allow us to explain the uremia which is due to the renal failure. Following the lead of King, who long ago showed that renal failure from a variety of causes was associated with decreased alkaline phosphatase; Dr. Robert Mowry and I were able to show histochemically that cases of renal failure of crush type, and other varieties as well, revealed a decrease or absence of alkaline phosphatase in the provincial convoluted tubules where the enzyme is naturally abundant (7).

The next slide (3) (frontispiece) shows the normal plentiful concentration of alkaline phosphatase in a section of the kidney, while that at your left shows the decrease and absence of the enzyme. The other section shows the similar loss of the enzyme in a case of malignant hypertension. In these two slides we have separated two types of the information which can be gained. The first slide allows the identification of the disease process, but does not allow us to say the cells are not functioning normally. The alkaline phosphatase preparation of the "crush" lesion permits us to say that one usual activity is missing in that disease process. While we do not know

what the alkaline phosphatase or phosphomonoesterase is doing or does, naturally we can say that it is normally present but absent or decreased in this and in very many other types of renal failure, or perhaps, all types.

The next slide is from a needle biopsy of the kidney from a human case of glomerulonephritis. It is stained by the PAS method and does not show any obvious abnormality. Because of the size of the sample obtained, I did not do the alkaline phosphatase preparation but a small portion of the cortex was fixed in osmium tetroxide solution and prepared for and studied by electron microscopy by my associate, Dr. Marshall Hartley. First I show you a normal portion of a glomerulus by electron microscopic methods. Please notice that each capillary wall contains, from within outwards, the reticulated endothelial cell, the lamina densa, and the covering podocyte cell. These three layers are to be examined in the next slide, the renal biopsy from membranous glomerulonophitis. The podocytes have lost many of the foot process, all are fused together in some area, and the lamina densa shows foci of thickening producing irregularity.

This is an example of a lesion which could be identified only by electron microscopy, the usual methods of light microscopy, including histochemistry, showing no obvious abnormality. At higher magnification the changes especially in the podocytes are more conspicuous, thanks to the superior resolution of the electron microscope. Let me add that instances in which electron microscopy shows lesions not recognisable by the light microscope are not very numerous in the case of kidney diseases.

The next few slides show the changes produced in the renal glomerulus by decrease in arterial blood supply, the condition which the pathologist calls ischemic. We call the condition ischemic obsolescence, meaning by this that the part is going out of action due to decreased blood supply. The changes to be discussed have recently been described by Dr. C. H. Lupton and me (4).

The first slide shows two glomeruli colored with the PAS stain. The smaller is quite abnormal, showing the wrinkling and thickening of the capillary basement membrane, while this change is focal in the larger glomeruli. These are the changes of ischemia and the next few slides show accentuation of these changes in the space of Bowman's capsule. The end result is shown in the next slide, a shrunken down knot of wrinkled capillary basement membranes surrounded by the hyaline material in the space of Bowman's capsule. The progressive changes in the glomerulus caused by ischemia are summarized diagrammatically in the next slide taken from McManus and Lupton (6).

The next slide demonstrates the appearance of and stage ischemic obsolescence as seen in a section colored with the routine hematoxylin and cosin stain. No details of the shrunken glomerulus can be made out nor can any glomerular remnant be recognized, while the next slide, colored with the PAS stain, reveals the hyaline knot to be the gravestone of the glomerulus. The histochemical technique again shows an unsuspected structural detail allowing identification of a lesion.

The next two slides show alkaline phosphatase preparations of the same hyline knot studied in the previous two slides. In the first of the phosphatase preparations, using glycerophosphate as substrate in the classical Gomori method, the hylaine knot shows little structural detail and no specific enzyme activity. However, in the next slide, using muscle adenylic acid as substrate (6) in a modified alkaline phosphatase histochemical method, the hyaline in the space of Bowman's capsule colors black, demonstrating high 5-nucleotidase enzymatic activity.

The significance of the presence of 5-nucleotidase in the hyaline filling the space of Bowman's capsule is not clear. At the present time we can say only that the specific enzyme is present and that this serves as a further factor or descriptor for ischemic change in the glomerulus. The next few slides show that the 5-nucleotidase appears early in the ischemic change in the glomerulus, perhaps in the covering epithelium or podocytes.

I show you again a normal glomerulus, as seen with the electron microscope, to recall to you the features of structure and the smooth roundness of the capillary wall. The next two slides are taken from pictures kindly lent me by Dr. Marilyn Farquhar. They show ischemic change in glomerular capillary loops, and, besides the wrinkly thickened lamina densa, they show also changes in the podocytes and in the endothelial cells which I shall describe in detail in a separate publication. To the best of my knowledge the late stages of ischemic obsolescence of renal glomeruli have not been studied with the electron microscope.

Reviewing what we have seen in the ischemic glomerulus, I should point out that we have indicated that the changes are seen best with a histochemical technique and; tracing the development, or "natural history," of the lesion, we have seen an early appearance of 5-nucleotidase in the ischemic glomerulus with the final shrunken glomerulus displaying a high concentration of the enzyme in the hyaline enclosing it. The electron microscopic appearances of ischemic glomeruli have been shown and described, I think, for the first time.

Turning to another glomerular lesion, intercapillary glomerulosclerosis, it is sufficient to say that this condition shows a constant association with diabetes mellitus. The first slide on a section from a typical kidney affected by this condition first described by Kimmelstrel and Wilson, and colored with the PAS stain, shows a concentration of a hyaline strongly PAS-positive material in the interluminal cell aggregates. The strong coloration by this method, which colors 1, 2 glycols chiefly, led me to suggest (2) that the glomerular hyaline deposits represented a mucoprotein from the blood trapped in the glomerulus in the process of excretion in the urine.

Electron microscope sections of the glomerulus in intercapillary glomerulosclerosis have been loaned me by Dr. Barber Muellar. They show a gradual progression

of the glomerular deposit to the final laminated knobs of the most marked condition. There is thickening of the lamina densa, which was not appreciable with light microscopy, even with the PAS method, from an early stage of the process. A feature of the condition of intercapillary glomerulosclerosis suggested by these electron micrograph preparations is that the hyaline may not all be the same within the glomerulus, here again differing from our histochemical PAS stain appearances.

Finally, note the changes in the ischemic glomerulus, and especially the 5-nucleotidase preparations. These are summarized in this slide from McManus and Lupton (5) while, as seen in this next slide, no significant 5-nucleotidase activity appears in the glomerulus at any stage of Kimmelstiel Wilson's intercapillary glomerulosclerosis. This absence is significant, since it has been said that ischemia is the cause of intercapillary glomerulosclerosis, a statement contradicted by the data presented here.

I have reviewed some of the biological aspects of the problems of pathology. I have also shown you some of the ways in which identification of lesions can be aided by histochemistry and by electron microscopy. In several instances of renal disease, the loss of an enzyme has been capable of indicating functional change. Contrawise, the appearance of an enzyme, where it should not be present, has aided in the identification of a lesion and the separation of one lesion from another. Finally, we have traced the "natural history" or development of some glomerular disorders, reminding us that lesions possess temporal qualities like all living activities. All these data have been presented as biological aspects of pathology.

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Quantitative cytochemistry for the study of normal and abnormal growth

TORBJÖRN CASPERSSON

Institute for Medical Cell Research, Medical Nobel Institute, Karolinska Institutet, Stockholm, Sweden

IN RECENT YEARS the possibility of studying the chemical basis of the metabolic processes that take place within the cells during normal function and under diseased conditions has radically improved. The cytochemical work usually involves a combination of biochemical and biophysical procedures which are sometimes rather complex. Different metabolic processes make different demands on technique. This applies particularly to the "resolving power", which is determined by the size of the individual objects to be investigated. These can be whole tissues, individual cells, large cell organelles, or individual small cell details.

In certain cases, it might be enough to use large portions of tissue for each study. For energy metabolism, as an example, it is often sufficient to work with homogenized samples of tissue, provided only that the cell populations in these are homogeneous. Work involving a higher degree of resolution is made possible by fractionation of the homogenized tissue before the chemical investigation by centrifugation, for instance, so that there is collected in the different fractions primarily one or another type of cell element.

These procedures have shown themselves valuable for the study of those structural elements that can continue functioning even when broken loose from the cell structure and a great deal of information has been obtained in this way on the details of the general catabolic processes, particularly with respect to enzyme function in the cellular material. The combination of such procedures with morphological studies, particularly with the use of electron microscopy, has given a wealth of information, e.g., on the mitochondrial and microsome functions.

Specialized cell functions such as the differentiation processes during cell development, demand as a rule work with a much greater resolution and this brings us into the field of cytochemistry proper. Qualitative cytochemistry, which works mainly with color-producing reactions, staining procedures and enzymological procedures, has yielded many results that are of great interest, in spite of the fact that many of the methods in this field have come in for some justifiable criticism.

The qualitative methods have their natural limitations and it is evident that development of quantitative cytochemical methods would broaden the field of study enormously. The first real advance here came with the study of enzymes, where a remarkable series of methods, primarily built up at the Carlsberg laboratory in Copenhagen, made possible great extensions of our knowledge of many normal cell functions. For quantitative studies of substances without enzymatic effects quite different types of methods are needed. Especially the efforts to study the synthetic or anabolic processes in the cell have been the reasons for their development.

The anabolic processes in the cell, especially those that lead to the building up of macromolecules, lead to very stringent requirements for a high degree of resolution in the cytochemical work. This group includes primarily the proteins, which comprise the main mass of the cell, but also many differentiation products that appear in cells during development and cell function.

Dealing with methods for the study of normal and abnormal growth processes, which is the task of this paper, the main emphasis has to be on methods for work on protein synthetic processes in the cell and their associated nucleic acid metabolisms. The main emphasis is placed, not upon the results won as yet, but on the type of information the quantitative methods can give; and the examples to be presented are, because of that, purposely chosen from widely differing fields.

For the work on growth processes, in which various cellular structural elements are concerned, it is on the one hand necessary to be able to work with a resolution down to about 1 μ because of the small size of some cell organelles. On the other hand, it is necessary to be able to make quantitative determinations of the substances in question in that dimension range. This is because of the fact that the main mass of the cell is comprised of proteins, together with varying amounts of nucleic acids, and the synthetic processes to be studied involve changes in the quantities of these substances in different parts of the cell.

The quantitative, cytochemical, high-resolution methods have primarily been developed for the study of just these questions and the available observation material is predominantly from this field. It should,

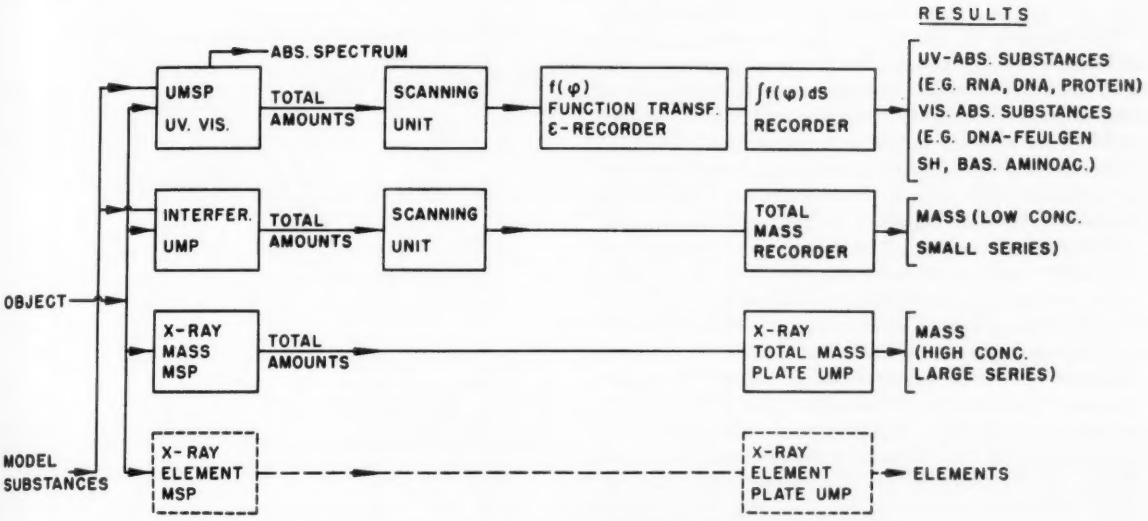

FIG. 1. Instrumentation for high resolution quantitative cytochemical work.

however, be stressed that the methods that are now available are perfectly suitable for the investigation of many processes of function and differentiation.

QUANTITATIVE CYTOCHEMICAL HIGH RESOLUTION METHODS

The first of these methods was developed quite long ago expressly for the study of nucleic acids in individual cell structures and with the aid of these methods there was demonstrated, among other things, the connection between nucleic acid and protein metabolism. Since those days the techniques have, of course, been developed considerably and there is now available for the study of protein and nucleic acid metabolism a series of interconnected biophysical methods by which accurate information can be obtained on the amounts of proteins and nucleic acids in cell structures measuring about 1 μ. The central methods are high-resolution ultramicrospectrography in the ultraviolet and visible spectral regions, as well as high-resolution microinterferometry and microradiography for the determination of mass (and in certain cases for S and P determination).

For truly quantitative work it is, as a rule, necessary to apply different biophysical techniques on one and the same object which tends to make the work technically complex. The present-day procedures are, however, so extensively automatized and standardized that they are well suited for use in most biological laboratories.

The reason why the quantitative cytochemical work on growth problems involves somewhat elaborate instruments lies in the fact that, for most rational types of biological problems, the instrumentation has to make it possible: *a*) to measure spots in the specimen down to 1 μ; *b*) to determine the total amounts of substances with known optical qualities in portions of cells or whole cells, in spite of the extremely great inhomogeneity usual

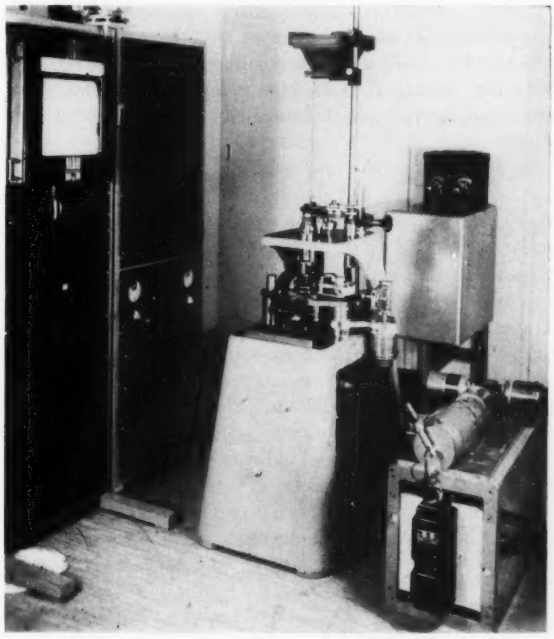

FIG. 2. The "Universal ultramicrospectrograph."

in the biological specimens; *c*) to control the optical conditions in the specimen to be measured; *d*) to determine in suitably arranged model systems the optical constants for the cellular substances of interest, under the conditions existing in the specimen being measured, which are usually very different from the conditions in true solutions; *e*) to check, in suitable objects, the cytochemical measurements against macrochemical measurements, in spite of the enormous difference in working

range of the two fields of work, which is around 10^6–10^9.

The necessity of meeting all the above requirements in biological routine work has led to the development of a relatively complex instrumentation, which can be said to have reached its definitive state in 1957, and which is described in detail in the literature (1, 2, 5, 6).

Figure 1 shows this system of basic instrumentation for general quantitative cytochemical work, which fulfills the requirements in the points mentioned above.

The diagram indicates the main procedures ("UMSP": ultramicrospectrography; "Interfer. UMP": high resolution interference ultramicrophotometry; "X-ray mass MSP": X-ray microspectrophotometry for mass determination; "X-ray element MSP": X-ray element microspectrography). In the figure the squares represent instruments. The right-hand column gives examples of the type of information obtainable with the different sets of instruments on the field now under discussion. For details and literature references see (2, 5).

The central instrument in the group is the UMSP, which is shown on Fig. 2.

Normal growth. Work carried out on normal growth in the past decade with the above methods and their predecessors has concerned various different aspects of the gene function mechanisms, nucleo-cytoplasmic interactions, the interplay between the courses of DNA, RNA and protein syntheses, and also certain differentiation processes. A highly varied cell material has been used for these studies by different working groups and a considerable bulk of information has been collected.

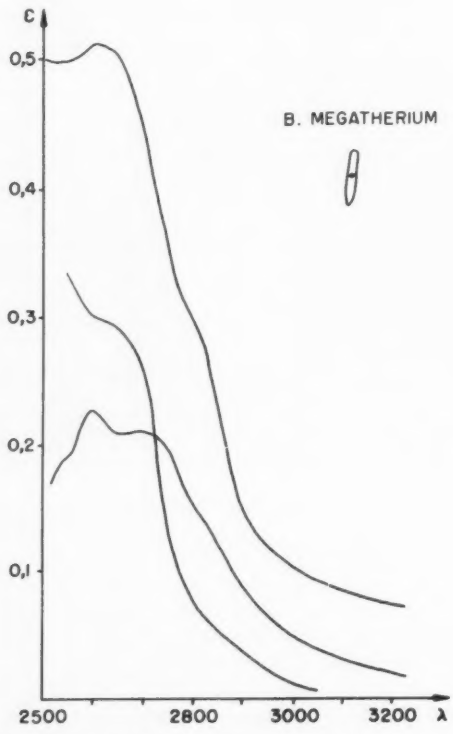

FIG. 3. Ultraviolet absorption spectra from individual bacteria in different stages of growth. The diameter of the bacteria is about 0.5 μ.

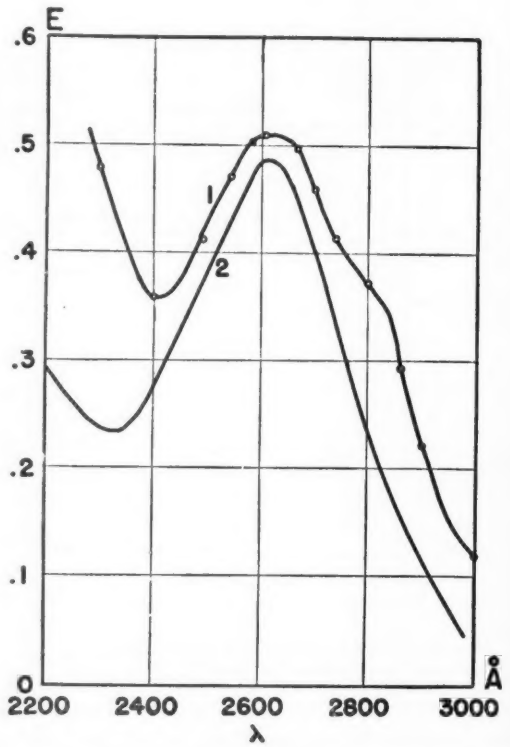

FIG. 4. *Curve 1:* Ultraviolet absorption spectrum of central part of a metaphase chromosome from a grasshopper, diameter about 0.7 μ. *Curve 2:* Course of the absorption of a solution of DNA.

This gives an opportunity to judge which are the most important cell parameters for the study of growth and also the extent to which these are technically accessible. Most of the cell nuclear organelles have a part to play in the protein synthetic processes. During metaphase the chromosomes consist of DNA and protein in very roughly equal proportions. On suitably chosen cytological material, it has been shown that, during the periods of growth and function, there appear in and around the gene loci both proteins and RNA, which are then transported out to the cytoplasm where the quantity of RNA can reach very high values during rapid growth.

Of particular interest in connection with growth is the behavior of the main nucleolar apparatus. The cytochemical studies show that this apparatus contains large amounts of proteins and RNA, which in all probability derive from the nucleolus-associated chromatin. The system of nucleolus-associated chromatin and main nucleolus seems to represent a special, very conspicuous mechanism for synthesis during growth of large RNA and protein masses of a relatively uniform type appearing in the cytoplasm. It is still uncertain in what part of the

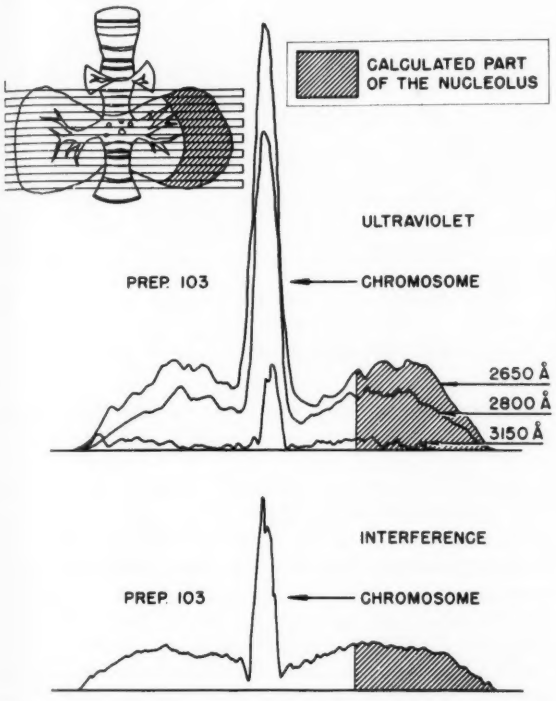

FIG. 5. Scanning high resolution measurements on a heterogeneous object.

cytoplasm the protein masses are to be found. Whether they represent structural protein, ribosome protein, or are diffusely distributed throughout the cytoplasm in order to serve as a basis for further rebuilding processes of the proteins, such as adaptive enzyme synthesis, is not known.

It is necessary, for practically any type of the work in this field, to be able to measure DNA, RNA, and protein in such small structures as met in the nucleus—in the magnitude 1 micron—and also to make determinations of total amounts of these substances in large and often extremely inhomogeneous cell parts.

A list of the different cell parameters, which it is of interest to measure in connection with growth problems, would thus run as follows:

1. In the chromosomes and chromatin: *a*) the DNA content per nucleus (and individual chromosomes), *b*) the RNA content, *c*) the protein content in the total nucleus.

2. In the nucleolus: *a*) the size, *b*) the RNA content, *c*) the protein content.

3. In the cytoplasm: *a*) the amount of RNA, and, eventually its distribution, *b*) the amount of protein.

All these cell parameters are well within reach with what was described above as the basic instrumentation. Illustrations below, taken from the work of different working groups in our institute, exemplify such measurements.

The lower limit in object size in ultramicrospectrography is set by the wavelength of the light used. On objects smaller than two times the wavelengths of the light used it is, for physical reasons, not possible to get detailed absorption spectra. Figure 3 illustrates that it is technically possible to reach close to this limit. It shows ultraviolet absorption spectra of bacteria in different stages of growth and thus with different RNA amounts in the cell body. The diameters of the bacteria are in all cases around 0.5 micron. Figure 4 shows the course of an absorption curve from a chromosome of similar size.

Biological objects with especially large cells give opportunities for studies of effects of individual genes or of gene groups. Figure 5 stems from a line of work on the synthesis of nucleolar substances in growth in *Chironomus* (11). Thanks to the enormous size of the chromosomes, it is possible to measure in detail the synthesis of nucleolar material from special chromosome regions. The figure also illustrates the scanning mode of measurement by which the influence of optical inhomogeneity in the object can be mastered. A small measuring spot is passed over the object in a scanning pattern and an electronic system simultaneously computes and records both the extinctions in the different points of measurement and the running integral values. The curves of four individual sweeps across the object (absorption measurements at 2650 A and 2800 A and interference measurement) are to be found in the figure. From the figure, it is clear how it is possible to make determinations of total amounts of absorbing and refracting substances also in discrete parts of a complex object.

The optical inhomogeneity is of specially great importance in measurements of large objects, such as whole nuclei and cells. However, the scanning measuring procedure, with measuring spots of the order of magnitude of the wavelength of the light, can account for such inhomogeneities in most types of biological objects. To take an extreme example—the largest individual cells used in our laboratory are protozoa, such as *Paramecium*. Here the cells are up to 200 μ in size and very inhomogeneous. In connection with studies of growth processes in *Paramecium* (8, 9), it was found possible to attain a very good reproducibility in measurements of individual whole cells (total mass, total nucleotides, and total DNA).

These few examples from different lines of work may illustrate how, with the presently available techniques, the cell parameters listed above can be analyzed on a variety of different cell types and thus growth problems of many different types can be studied, in both normal growth and in diseased conditions. The latter field has not been worked on much as yet, excepting tumor growth, but the potentialities here are great.

In this connection, it should also be pointed out that the present methods are well suited to studies in other fields of interest for cellular pathology, namely cell differentiation and cell function. Little work has been done here as yet, but the results indicate that the field will prove to be a fruitful one. As examples, the following have been studied: The function of various glands, the

reaction of some tissues to hormone stimulation, amyloid development, the keratinization process in normal and pathological cases, and, lately, the mast cell system by aid of quantitative evaluation of metachromatic and other staining reactions.

In a general way, it can be said that the group of combined absorptiometric and interferometric procedures working with the apparatus referred to in the beginning as basic instrumentation for more general cytochemical work have proven to be suitable for the study of quite a wide variety of problems in normal and abnormal cell function.

Abnormal growth. The most interesting contribution of quantitative high resolution cytochemistry, as yet to our knowledge of the abnormal growth, is the observation of a great cytochemical variability between the cells of malignant cell populations with regard to organization and composition of the cell organelles involved in the protein and nucleic acid synthetic processes. This seems at the moment to be the most distinct chemically definable difference between normal cell populations and malignantly growing ones. In general pathodiagnostic work, cell polymorphism is a well-known sign of malignancy. The stained preparations, however, judged by the eye, reveal mainly morphological structure changes or

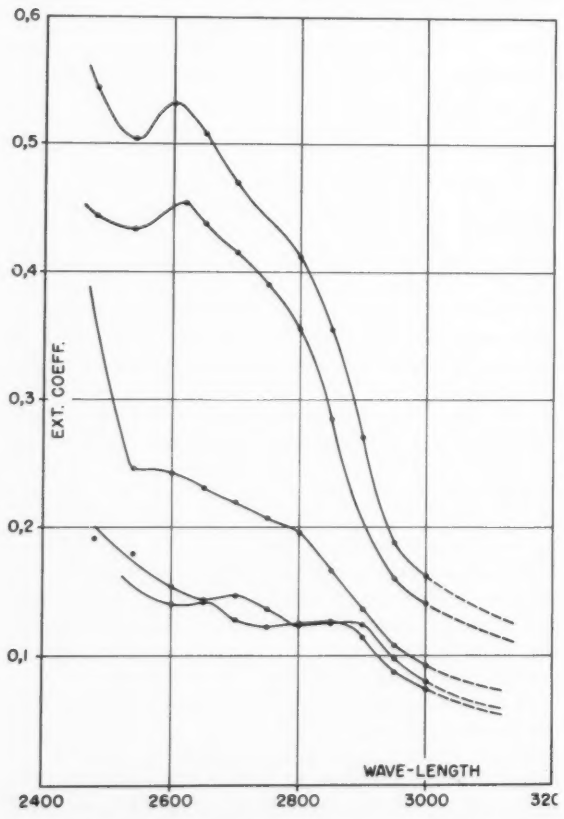

FIG. 6. Ultraviolet absorption spectra from small spots in cells from different parts of a solid human carcinoma.

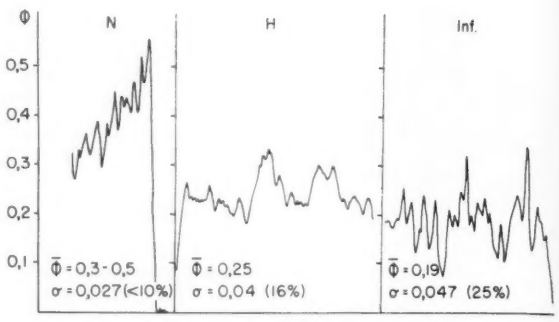

FIG. 7. Dry mass variability between cells.

changes in amount and distribution of the products of cell differentiation and function, which occur secondary to the fundamental changes in growth pattern.

Already the early cytochemical studies of the protein and nucleic acid metabolism with quantitative tools—the first some 20 years ago—showed striking differences between individual tumor cells (7, 10). These differences were especially pronounced in cell parameters concerning the RNA and cytoplasmic proteins but were found also in the DNA. No penetrating study of the subject was possible with the methods then available, so that the field lay for a long time untreated because of the technical difficulties involved. Investigations with the more recent general procedures for RNA-, protein- and DNA-determinations in our institute, and recent studies with the Feulgen reaction from several other laboratories, have shown that the "cytochemical variability" is a very prominent feature in malignant tumor growth.

This variability concerns practically all the parameters discussed earlier in this Symposium, and can be extremely pronounced in some types of tumors. Figures 6 to 8 give some examples from measurements on solid invasive tumors. In Fig. 6 are plotted UV-absorption spectra of small spots in cells from different parts of a solid human carcinoma. The curves show very great differences in the concentration of cytoplasmic RNA, and indicate, also, differences in protein concentration. The curves in Fig. 7 show microradiographic mass measurements around and in a skin carcinoma. The measuring spot has in each case swept over about 10 cells. N is normal tissue, H the hyperplastic tissue around the tumor, and Inf. the infiltratively-growing tumor region. The recording from normal skin reflects the small variability in the protein mass between adjacent cells. The increase from left to right is caused by the lower water content in the keratinized surface cells of the epidermis. The hyperplastic region shows lower mass values and somewhat larger variability, while the tumor region shows a very irregular recording, reflecting very great differences between adjacent cells. Figure 8 from the same preparation shows the very conspicuous changes in the nucleolar apparatus (optical cross-sectional area). The uppermost curve is from the normal skin, and the lower from regions closer and closer to the infiltratively

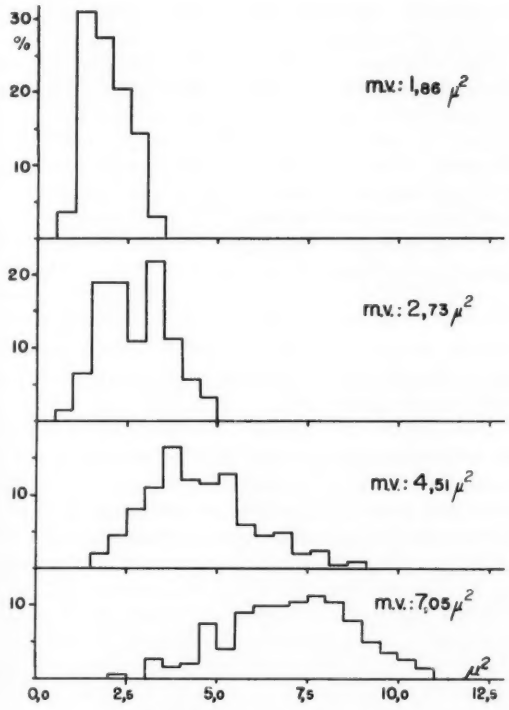

FIG. 8. Variability in nucleolar size.

growing part. There not only occurs an increase in average size, but also a very pronounced increase in the variability in the amount of nucleolar substances.

The phenomenon of cytochemical variability is of equal interest for tumor therapy, carcinogenesis, and certain tumor diagnostic problems.

Interest from the tumor therapeutical point of view stems from the fact that a cell population with a great variation in the organization of the cell organelles involved in protein and nucleic acid synthesis obviously can be expected to react on external agents in patterns different from those of a normal homogeneous population.

For studies of carcinogenesis, it is of interest to investigate the stage in which these variations between cells appear. Such a study should also give valuable information for the judgment of early cellular changes in diagnostic work.

Work on these lines has, to a certain extent, been carried through with the techniques mentioned above. For instance, it was found in a mouse ascites tumor strain, which regularly developed resistance to methotrexate during treatment, that the DNA-distribution pattern in all resistant strains studied showed a similar marked deviation from that of the original tumor, clearly indicating a differential sensitivity among the cells (see 4). Recent observations with other anticancer agents have given additional evidence on that point.

In connection with the work on effects of external agents, relatively comprehensive studies have been made on X-irradiated ascites material (3). It was found that DNA-, RNA-, and cytoplasmic protein synthetic processes responded in different ways. This possibility of differentiating on a cytochemical level between the reaction of these different metabolic processes is also of considerable interest for work on chemotherapy.

Extensive studies have been made on surface biopsy material from human cervical carcinomata in different stages of development (see 4). It was found that in the established carcinoma—as compared with the normal mucosa—there appear very pronounced changes in the variability pattern of the cytoplasmic RNA and protein and also of the DNA. In limited material from "in situ" situations, changes in the degree of variability were also observed.

RAPID CYTOCHEMICAL POPULATION STUDY METHODS

The observations referred to above, supplemented by some other similar studies, showed the cytochemical tumor cell variability to be a still more conspicuous phenomenon than earlier studies had given reason to believe. Thus, it was desirable to expand the population studies. The recording cytochemical techniques described above proved, however, too slow to permit study of very large populations.

The possibility was therefore considered in 1958 of evolving rapid population study techniques. This would clearly mean a very considerable amount of biophysical construction work, plus a great deal of biological work on the selection and development of suitable types of cell material. In cooperation with Doctors Sidney Farber and George Foley at the Children's Cancer Research Foundation in Boston, and a group from our institute, this question was examined thoroughly, and it was judged to be worthwhile to carry through such a project, especially because of its interest in tumor therapy. The biophysical instrumentation development was carried out in Stockholm in 1959 and 1960, while the development of biological material was simultaneously handled in the American laboratory. During 1960, the instrumentation has been used for work on the fields mentioned above. It is at present under publication (T. Caspersson, S. Farber, G. Foley, G. Lomakka, D. Killander, O. Caspersson).

It should be pointed out that new instruments have increased the speed of the population work by 100–500 times on suitable material. This changes the whole background for cytochemical population work and makes much wider problems than before accessible for study. Studies are at present under way on different problems concerning carcinogenesis and chemotherapy.

In order to make this great gain in speed possible, methods had to be limited to types of material where the cells could easily be isolated from each other. For certain questions this is no disadvantage. For others, however, it is desirable to have access to rapid techniques and also to work in solid tissues. Such methods are at present

under development but they offer considerably greater technical problems.

SUMMARY

The high resolution quantitative cytochemical procedures available at present are well suited for study of many aspects of normal growth involving changes in the state of the intracellular structures concerned in the process of protein synthesis in the cell and, also, for the study of different types of growth disturbances.

For the study of cell differentiation and cell function, the automated techniques have considerable research potentialities which have by far not been fully utilized as yet. In the field of tumor growth and differentiation, the most interesting contribution thus far has been the observation of the wide cytochemical variability in different cell organelles arising during tumor development and becoming most pronounced in malignantly growing cell populations.

This variability is a factor of significance for the mode of reaction of tumor cell populations to external agents, such as chemical anticancer substances. It is also of interest for questions concerning carcinogenesis and diagnosis in early stages of tumorogenesis. As these fields seem to be especially promising for the future, rapid cytochemical methods have lately been developed specially for populations studies.

With regard to the quantitative cytochemical work on growth in general, it seems probable that the next few years will see an increasing use of biophysical methods for that purpose in cell physiology and cellular pathology. A factor which has until recently limited the use of such methods has been the relative complexity of the instruments and the attendant difficulty of access to suitable instruments. Both these factors, however, have changed rather radically during the last years. The basic instruments are—and will necessarily remain—rather complicated, when compared with many other tools of cytology, but the routine work with them is nowadays simple. Commercial production of some types of instruments is in progress.

Thanks to the existence of the more complex and precise instrumentation, it will be also possible in an increasing scale to attack problems in a quantitative fashion by simpler procedures, calibrated and controlled by aid of the more complex techniques. Population study techniques give one such example. No doubt many such lines will be developed in the future. One field, which will obviously benefit from such an approach, is the study of living cells.

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Vistas in the etiology and pathogenesis of tumors[1]

JACOB FURTH

Department of Experimental Pathology, Roswell Park Memorial Institute, and Graduate School, University of Buffalo, Buffalo, New York

TECHNIQUES OF QUANTITATIVE absorption-spectroscopy for nucleic acid were introduced by Professor Caspersson. These and other cell analyzers, such as modern chromosomology, are excellent tools for characterization of the cancer cell and cell populations. They support the classical concept (i.e., that the core of the cancer problem is the cancer cell, which is a permanently altered autonomous variant of a normal cell), which was the subject of the Symposium of the American Association for Cancer Research (Cancer Research in print, and 19).

In this Symposium, James Miller reviewed the mechanism of carcinogenesis by chemicals on the molecular level, from the binding of the carcinogen through a series of reactions ultimately leading to a modification of the cells' hereditary nucleic acid. Miller, as well as others, postulates a basically similar change in nucleic acid by viruses and radiations. While true for some, this postulate does not explain the origin and character of all cancers. I wish to balance these concepts by tracing many neoplasms to a disturbance of the homeostatic equilibrium which specifically regulates the number of each cell type of a complex organism. I shall describe progressively growing tumors, where the basic change is not in the cells to be regulated, but in the regulatory apparatus and this change is readily correctible.

To illustrate the role of specific homeostatic regulators in the initiation and maintenance of growth of many tumors, two organ systems were chosen: The Mg–Mt and the T–Tt systems.

Abbreviations:
T = thyroid; Tt = thyrotrope; TtH = thyrotropin—thyrotropic hormone; TT = thyroid tumor; TtT = thyrotropic tumor.
Mg = mammary gland; Mt = mammotrope; MtH = mammotropic hormone = mammotropin; MtT = mammotropic tumor; MT = mammary tumor.

[1] Supported by the National Cancer Institute and the Atomic Energy Commission.

The title of this communication was suggested by our chairman. Vista is defined as a "mental view of far reaching kind." This is too pretentious. Vista is also defined as "A view, especially one seen through a long narrow avenue, as between rows of trees." In cancer research we are passing through a long and narrow avenue lined with tall "trees" limiting our horizon.

MAMMARY TUMORS—MAMMOTROPES

The discovery of hormone responsiveness of tumors can be traced to Beatson who, in 1896, by bold reasonlng based on the relationship of breast cancer and milk production to ovarian function in the dairy cow, recommended removal of the ovaries for control of breast cancers in women (3), a procedure which is almost routine today. Other discoveries followed, as indicated in Table 1.

Subsequently, work by many investigators has led to the concept of a triad of forces in carcinogenesis:

1) Carcinogens, which are agents that bring about an apparently irreversible modification of the cell's hereditary nucleic acid with loss of ability to respond properly to homeostatic regulators.

2) Hormones, which are promoters and inhibitors in cell homeostasis. They regulate growth and function of cells but do not produce cancers. If cancer arises in a hormone stimulated organ, we had better search for another force.

3) Genetic factors, which govern the response of the host to extrinsic agents. They do not operate in a vacuum. The discovery that MT and leukemia in certain strains of mice is due to viruses dims the luster of those genetecists who published papers on the number and character of genes involved in the high frequency of these cancers. This dilemma can only be resolved by accepting the dictum that genetic material and viruses are the same (18).

All three types of carcinogens (chemical, viral and radiation) have been demonstrated to be capable of inducing mammary tumors. The hormones responsible for MT were first thought to be estrogens, thus explaining why breast tumor is common in women and rare in men. However, in regulation of mammary gland function, estrogens act indirectly by way of the pituitary Mt, stimulating the production of mammotropins (MtH) which, in turn, stimulate the mammary gland. Some 10 years ago, while working on radiation-induced cancers, we were accidentally sidetracked to a study of the mammotrope. When the pituitary is irradiated, the mammotropes readily give rise to functional tumors which can be grafted on normal hosts, causing an extraordinary stimulation of the mammary gland, even in

TABLE 1. *Historical Data Related to Control of Breast Cancer*

1896	Ovariectomy retards MT in women (Beatson, 3).
1916	Prevention of MT in mice by ovariectomy (Loeb).
1932	Estrogen induces MT in mice (Lacassagne).
1939	Adrenal compensates for ovariectomy, produces gonadal hormones and MT (Fekete, Woolley and Little, 23).
1940	Discovery of milk virus (Bittner, 5).
1944	Androgens inhibit MT development (Heiman).
1951	Inhibition of MT in women by adrenalectomy (Huggins and Bergenstal, 13).
1952	Hypophysectomy for control of MT in women (Luft and Olivecrona, 17).
1958	Isolation of Mt. Its relation to estrogen; its role in genesis and growth of MT (8).

Concerning effects of hormonal therapy of human MT see 20.

FIG. 1. Extensive mammary gland stimulation of a rat bearing a transplanted mammotropic tumor in the right thigh.

the absence of the gonads (8). Figure 1 shows the extensive mammary gland stimulation in a rat bearing a grafted mammotropic tumor in the thigh.

In studies on induction and growth of mammary tumors, such transplantable mammotropin secreting tumors were used to continuously supply large quantities of MtH in order to study its role in the initiation and growth of mammary cancers induced by three classes of carcinogens: chemical, viral and physical.

Mammary tumor induction with radiations. It has been established that radiation readily induces mammary tumors in rats (6). In a typical experiment, rats were given 50–400 r of X rays (Table 2). Within the 8-month period of observation, no MT developed in the rats treated with MtH or with 50 r alone and only 6% of the rats given 150–400 r developed such tumors. In contrast, 58 per cent of the hormone treated rats irradiated with 50 r had mammary tumors and larger doses increased the tumor induction rate to 72% (24, 25).

Recent evidence links induction of leukemia by radiation to activation of a latent virus—but viral action

TABLE 2. *Role of MtH in Induction of MT by X rays in Rats (24, 25)*

Treatment	Tumor Incidence
50 r	0/14*
150–400 r	2/35 (5.7%)
MtH	0/21
50 r + MtH	7/12 (58.3%)
150–400 r + MtH	24/33 (72.7%)

* Number injected over number with tumors.

TABLE 3. *Role of MtH in Induction of MT in Mice by Virus*

Treatment	No. in Group	No. with Tumors
MtH + virus	37	12 (32%)
Estrogen + virus	30	0
Virus only	30	0
MtH only	39	0
Estrogen only	30	0
None	30	0

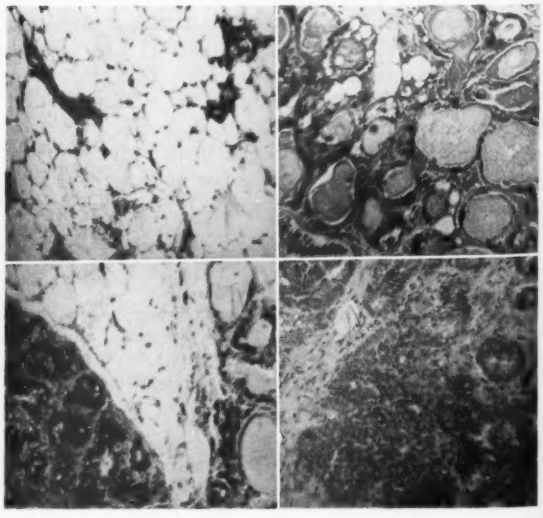

FIG. 2. Microscopic appearance of the normal mammary gland of a female mouse (left upper quadrant) contrasted with mammary gland stimulated with mammotropic hormone containing much inspissated secretion (right upper quadrant), similarly stimulated mammary gland with a micro-tumor (left lower quadrant) and of a large carcinoma (right lower quadrant).

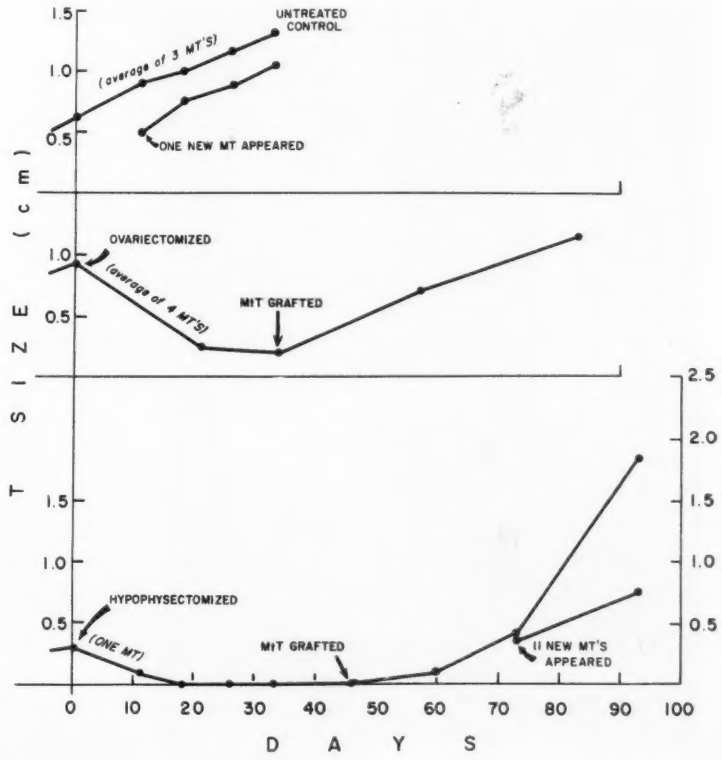

FIG. 3. Inhibition of mammary tumors in rats induced with 3-methylcholanthrene by ovariectomy and hypophysectomy and resumption of tumor growth following administration of mammotropin.

itself is explained by a secondary nucleic acid disturbance. However, it is not necessary to suppose that radiations, which can directly modify nucleic acid, always act by activating a virus. Thus far, no virus has been found in MT of rats, although two viruses are known which are capable of producing MT in mice. Let us examine, therefore, the possible role of Mt in induction of MT in mice by virus.

Role of MtH in induction of MT by virus. The mammary tumor virus of Bittner is highly infectious during the first few days of life. Adult mice are resistant. In the following study (Table 3) (24, 26), adult mice of the LAF_1 strain, in which mammary tumors are almost nonexistent, were used. Of the 37 mammotropin stimulated mice treated with virus, 32% developed cancers; on the other hand, in 159 mice that received virus or hormone alone, or estrogen with or without virus, none developed tumors.

What is behind this remarkable phenomenon? The present popular concept links tumor formation with maturation arrest and with disturbance of differentiation. According to this concept, a nondividing end-cell cannot form a tumor and when a cell turns tumorous, it multiplies without perishing by maturation. In the genesis of MT and TT discussed here, differentiation is not involved. The specific hormones appear to have done no more than bring about proliferation of a fully differentiated cell. Whoever has seen a motion picture of mitosis with the extraordinary pinocytic movements, can easily visualize how viruses can gain entry into cells during mitosis. If one asserts that virus is the sole cause of cancer, other factors, without which the virus is harmless, may be ignored. If the tumor virus is ubiquitous, its mere identification is almost meaningless. What becomes important is to determine why the ubiquitous virus causes cancer in one individual, but not in another.

Figure 2 contrasts the normal obese breast with the highly hormone-stimulated mammary gland containing much stagnating secretion, an early tumor, and an invasive cancer.

Electron micrographs, just completed by Dr. Leon Dmochowski, have shown an abundance of virus particles in the virus-induced MT but not in the MtT.

Role of MtH inducing MT with chemical carcinogens. Several investigators (22) have shown that feeding female rats with carcinogenic hydrocarbons will cause the development of many mammary cancers. Mammary cancers were induced in rats (15) by repeated feeding with 3-methylcholanthrene, as reported by Huggins (14). When the tumors were palpable, the rats were arranged in groups of three: one was left untreated as control, the second was ovariectomized and the third hypophysectomized (Fig. 3). In about two-thirds of the ovariectomized or hypophysectomized rats, the tumors diminished in size and some completely regressed. When mammotropin was administered to such rats (indicated by the arrows), the atrophic tumor rapidly resumed vigorous growth.

FIG. 4. This rat had a mammary tumor induced by 3-methylcholanthrene which regressed following hypophysectomy. On subsequent administration of mammotropin, not only did the original tumor recur but numerous new mammary tumors appeared, as shown.

In such heavily cancerized hypophysectomized rats, not only did the original tumor recur following MtH treatment but many new tumors appeared, 11 of macroscopic size in the rat shown in Fig. 4, with a host of microscopic tumors. These observations indicate that the chemical carcinogen brought about a neoplastic transformation of many cells, most of which remained latent until stimulated by MtH.

In the experiment shown in Fig. 5, rats were given one 10 mg dose (oral) of methylcholanthrene. This, or MtH alone, produced no tumors. However, MtH treatment preceding or following administration of the carcinogen caused rapid development of mammary cancers in most rats. The hormone treatment could be delayed as long as 8 months (16), indicating again that cancer cells can remain latent over long periods, and can be made to grow when a proliferative stimulus is applied.

This phenomenon is not unique. It was shown in skin carcinogenesis with tar, decades ago, by Rous (21). By simply punching holes in a tarred area and thereby creating a proliferative stimulus, tumor formation was incited. This led to the two-stage concept of carcinogenesis systematically worked out by Berenblum (4).

Figure 6 contrasts the resting mammary gland of a female rat with that stimulated by MtH; an early cancer induced by methylcholanthrene, and a fully developed cancer in a rat treated with both.

To recapitulate, carcinogens initiate, i.e., cause an "irreversible" change in mammary epithelium. Promotion of growth of many altered cells, while still remaining under specific hormonal control, is later brought about by MtH. This hormone increases the number of cells in the target area, and thereby brings more cells in a state sensitive to carcinogens, particularly if it is applied before the carcinogen. Furthermore, the MtH stimulated gland may trap carcinogenic chemicals or pick up virus by pinocytosis or other mechanisms during division.

We conclude that the mammotrope is the key cell in the initiation and maintenance of mammary cancers and, therefore, also the key cell in its control. Figure 7 is an electron micrograph of Mt acidophils, left tumorous, right from the pituitary of a lactating female (7). Such large (100–200 mμ) electron dense particles (stored hormone) are absent in all other types of pituitary cells.

The concept of two types of acidophils, one secreting growth hormone, the other MtH (prolactin), is challenged by our observations. It seems to us more likely that the cell shown here secretes either one hormone with the two main effects or two closely related hormones.

THYROID-THYROTROPE SYSTEM

At present, this system provides the best model to illustrate how a homeostatic derangement can lead to progressively growing tumors and how such tumors can be fully controlled by restoration of the homeostatic disturbance (11).

High levels of thyroid hormone depress the pituitary thyrotropes; low levels of thyroid hormone stimulate thyrotropes (Fig. 8). In turn, the thyrotropins produced stimulate the thyroid and the increased blood level of thyroid hormone causes depression of thyrotropes. This is the thyroid-thyrotrope "thermostat." The situation

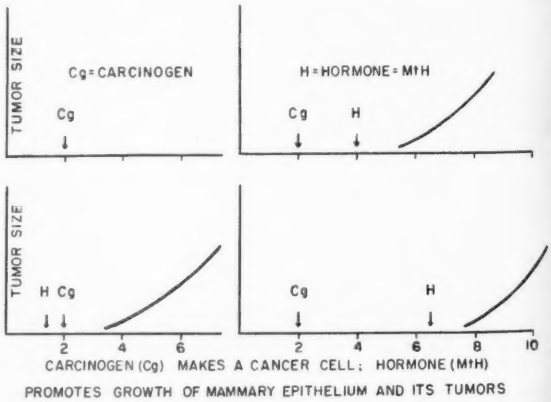

FIG. 5. Schematic presentation of the finding that a small dose of carcinogen alone produced no mammary tumor; the same dose produced tumors in animals also treated with mammotropins; and that the latency of tumor growth depended on the time of administration of this hormone.

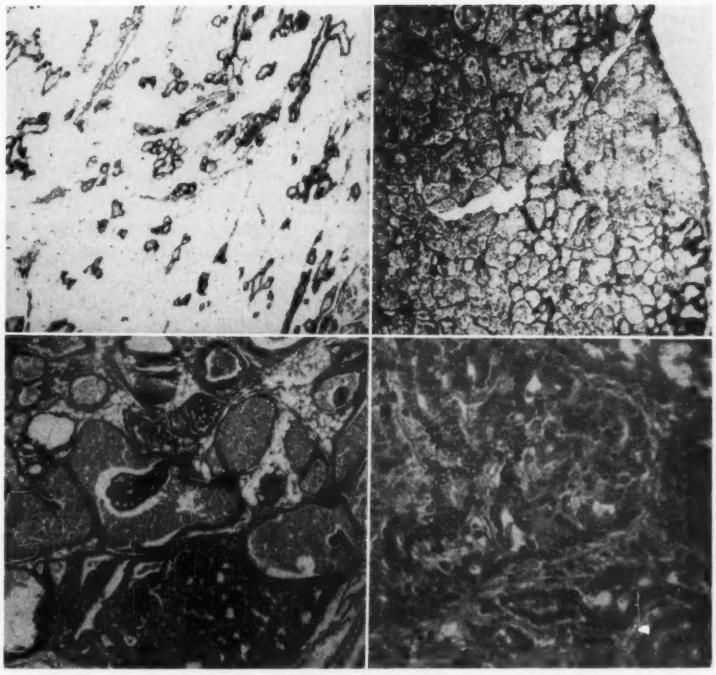

FIG. 6. Mammary gland of a normal adult female rat (left upper quadrant), that stimulated with mammotropin (right upper quadrant), an early microscopic tumor in a hyperstimulated mammary gland (left lower quadrant), and a large carcinoma (right lower quadrant).

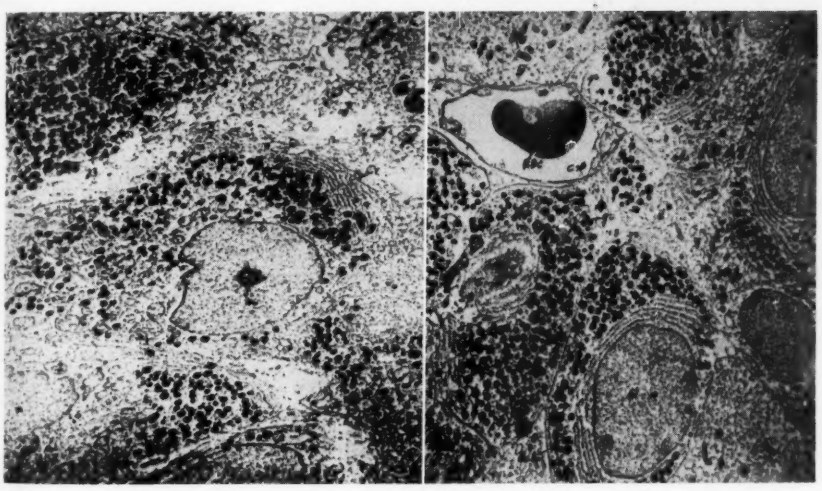

FIG. 7. Electron micrograph of neoplastic mammotropes (left) compared with acidophilic cells of the normal pituitary (right) (×7,300). (Courtesy of Dr. M. Farquhar and the M. D. Anderson Hospital.)

is more complicated than shown here. The signal substance to the pituitary may be a metabolite. The hypothalamus acts as the modulator, but the system works in absence of the hypothalamus, as was noted in the course of transplantation studies with fully hormone dependent thyrotropic tumors.

It is possible to produce at will either a thyroid tumor or a thyrotropic pituitary tumor by appropriate derangement of this system. Thyrotropic pituitary tumors are best produced by complete destruction of the thyroid with I^{131}, thus creating a sustained need for thyrotropic hormone. Conversely, thyroid tumors are produced by creating a sustained oxcess of thyrotropic hormone. (Fig. 9).

Homeostasis is complex and poorly known but the change can be often located and corrected. It is the balance that matters. Examples of several possibilities are listed in Table 4.

There is a striking difference between radiation and hormone induced tumors. With rare exceptions, tumors arising in radiated cells are autonomous from the start, while hormone induced tumors in the beginning are hormone dependent. However, if radiation hits the regulatory apparatus, as is the case with radiation in-

duced thyrotropic tumors, neoplasms which arise in the regulated cells are as frequently hormone dependent as those induced by simple hormonal imbalance.

Thyrotropic (pituitary) tumors (TtT, cf. 10). Sustained thyroid hormone deficiency in mice, best achieved with radiothyroidectomy, will invariably result in the development of a progressively growing pituitary tumor (Fig. 10). These tumors often invade the sella and adjacent brain.

When grafted, they never grow on isologous normal hosts, while they thrive in hosts conditioned by TH deficiency. They metastasize to regional lymph nodes, but even the metastatic tumor cells are fully hormone dependent. Grafted thyrotropic tumors usually remain fully dependent on TH deficiency for several years of successive passages in animals. However, sooner or later they give rise to highly autonomous variants. The morphologic changes accompanying this functional alteration have been amply illustrated elsewhere (cf. 10). In general, dependent tumors resemble a population of normal cells, while autonomous tumors are beset with the chromosomal (DNA) abnormalities, such as "anaplasia", discussed earlier by Professor Caspersson.

The characteristic change occurring in the course of progressive neoplastic transformation consists in an increase of growth vigor and decrease in functional capacity. The latency of primary TtT is about 16–10 months, that of dependent tumor grafts 8–4 months, and that of autonomous tumor grafts 3–1 months (Table 5). As the tumor acquires autonomy, function gradually decreases, as indicated in units of TtH per milligram of tumor.

Thyroid tumors. Functional thyrotropic pituitary tumors, providing continuously very high levels of thyrotropins, were utilized in attempts to produce transplantable thyroid tumors. My associates (11) succeeded in producing such a tumor, which even now, after 3 years of successive animal passages, is still fully dependent on thyrotropins.

The situation is analogous with that in man. Thyroid cancers are rare and most of them are highly dependent on deficiency of TH, which is their physiologic inhibitor.

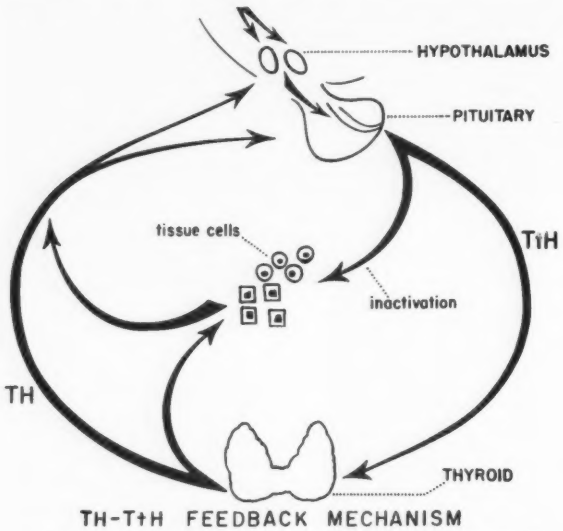

FIG. 8. Schematic view of the thyroid-thyrotrope "thermostat."

TABLE 4. *Homeostatic Disturbances Promoting Tumor Formation*

Stimulation can result from	Examples
Reduction of inhibitor	(Thyroid) TH → (pituitary) TtH +
Increase of stimulant	TtH → TH +
Diminished inactivation of inhibitor	Liver disease → estrogen +
Altered responsiveness of target cell	Radiation → all cells
Altered responsiveness of regulator cell	Radiation → all cells

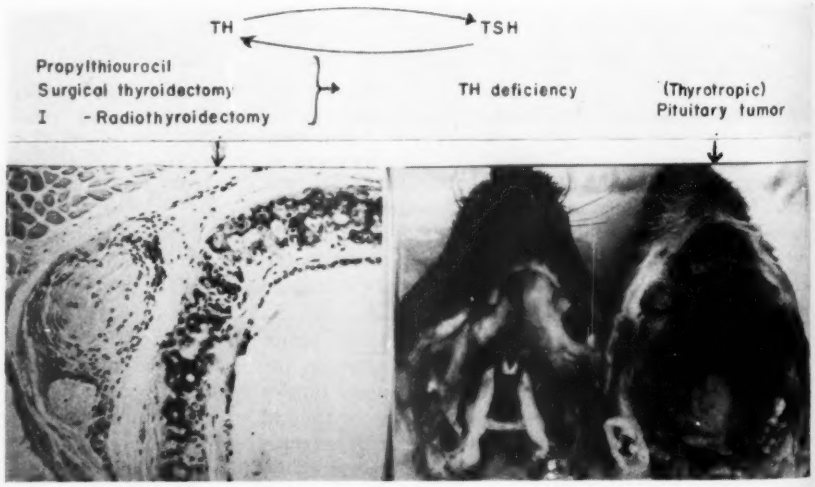

FIG. 9. Thyrotropic pituitary tumor induced by radiothyroidectomy contrasted with a normal pituitary. The photomicrograph shows the site of the thyroid region following radiothyroidectomy.

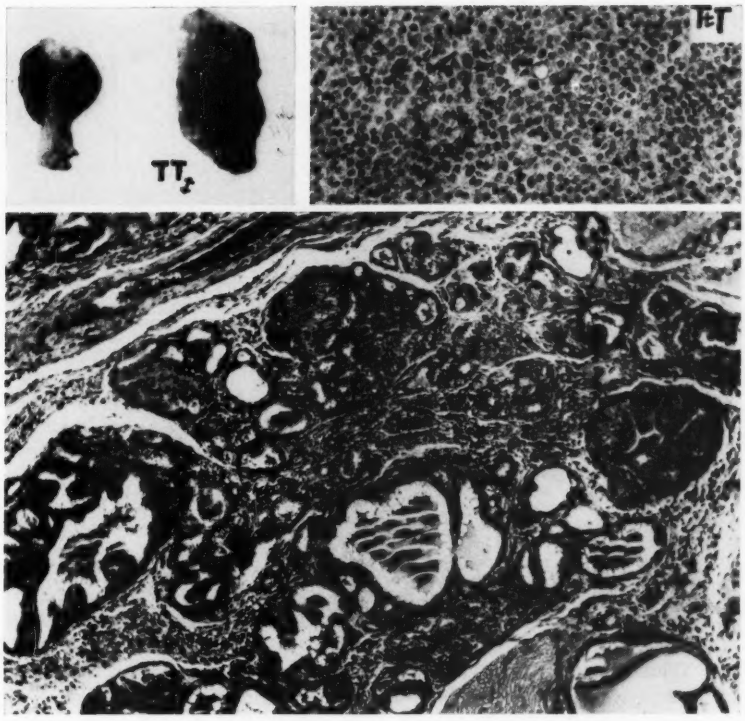

FIG. 10. Macroscopic view of a hyperstimulated thyroid and fully dependent thyroid tumor, with a microscopic picture of the latter.

TABLE 5. *Growth vs. Function—Thyrotropic Tumors*

	Growth	Function
	Latency months	TtH* content
↓Normal pituitary		0.1
Primary dependent tumor	16–10	0.4–0.05
Grafted dependent tumor	8–4	0.2–0.02
Grafted autonomous tumor	3–1	0.01–0.0

Scheme based on experiments of Bates et al. (2) and several members of my laboratories (11). * In units/mg; approximate values.

TABLE 6. *Growth vs. Function—Thyroid Tumors‡*

Thyroid cell	Growth*	Function†
↓Normal, resting	0	+++
Normal, hyperstimulated	+	++++
Dependent tumor	++	++
(Autonomous tumor	+++	+-0)

* Growth is measured by size, tumor latency, uptake of H^3-thymidine and mitotic figures. † Functional capacity is measured by I^{131} uptake. TH production. ‡ Schematic. Signs in parentheses are based on work of others.

(The common causes of this deficiency are lack of iodide or antithyroidal chemical in the diet.) Many thyroid tumors of man can be controlled by administration of thyroid hormone (1).

The behavior of TT has often fooled pathologists. A tumor cell population, as shown in Fig. 10, resembles a malignant growth. They can be seen in the lymph node,

TABLE 7. *Growth vs. Function—Thyroid Tumors*

Thyroid	I^{131} uptake, cpm/mg*	Weight, mg
↓Normal, resting	8800	5
Normal, hyperstimulated	3900	66
↓Dependent tumor	571	84
†(Autonomous tumor	500 → 0	100+)

* Values from an experiment of Messier-Furth (11).
† Schematic.

venules, draining the thyroid, and, rarely, in the lung, yet often they are not autonomous cancers. (Chromosomal changes have not yet been studied in the course of the gradual transformation from normal to autonomous cells. We would expect that in dependent tumors the chromosomes would be unaltered.)

The inverse relationship between growth and function evident in the course of thyroid tumor induction is sketched in Table 6. Although the transplantable thyroid tumor studied is fully dependent on thyrotropins, its growth vigor has increased and its functional capacity decreased, as shown in Table 6.

More precise documentation of this dissociation is shown in Table 7. Function, as measured by iodine uptake, decreased per mg of tissue, while growth vigor, indicated by tumor weights, increased with tumor formation. By multiplying the two figures in the vertical columns of Table 7, one arrives at the total I^{131} uptake. There is a gradual transition between the normal gland and the autonomous tumor. Hyperstimulation of the thyroid is at first diffuse; then nodules arise, many of

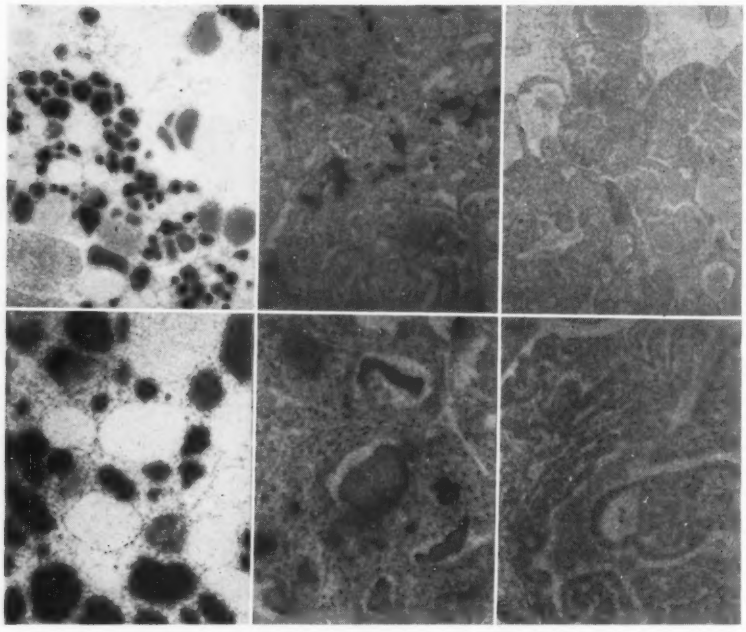

FIG. 11. Radioautographs of a normal thyroid (left columns), that of a hyperstimulated nodular thyroid (center columns), and dependent thyroid tumors (right columns) given the same tracer dose of I^{131} and sacrificed at the same time (courtesy of B. Messier).

which take up very little I^{131}, hence the decrease per mean unit weight. As one would expect, the functional capacity of both dependent and autonomous tumors tend to decrease in the course of successive passages.

Radioautography is an excellent means to illustrate both function and growth. Those of Fig. 11, made by Dr. Messier shortly after I^{131} administration, illustrate the decrease in I^{131} uptake with formation of adenomatoid hyperplasia and dependent tumor formation. The first vertical pair is from normal thyroid, the second from hyperstimulated nodular thyroid, and the third from a dependent thyroid tumor. In the last, the grains are fine, barely detectable, at this magnification.

TUMOR-HOST RELATIONSHIP

This now popular topic includes: *1*) specific hormonal factors and homeostatic regulators; *2*) nutritional factors, such as marked reduction of caloric intake and specific vitamin and other deficiencies, as both are known to be capable of retarding tumor growth; *3*) specific immunological forces (humoral and cellular), which may limit progression of spontaneous tumors; and *4*) miscellaneous nonspecific factors, such as ability of the host to form supporting stroma (connective tissue and blood vessels), provide macrophages and mast cells which may influence tumor development, and such poorly understood forces as the ability of the spleen and muscle to check proliferation of tumor cells, which often reach these organs by way of the blood stream.

Points 2,3 and 4 are possibilities which to date have been found to be of limited significance. Knowledge of vital anabolic pathways and of essential nutrients of cells may lead to exploitable differences between cancer and normal cells. For example, the unusual dependence of gonadal stimulation on folic acid led Hertz (12) to the discovery of the great responsiveness of choriocarcinoma of women to the folic acid antagonist amethopterin and to spectacular control of this tumor by high levels of this folic acid analogue.

However, ideas under *1* offer, we believe, as much promise as any for the control of cancer (9). The possibilities in hormonal control of neoplasm include: *1*) prophylaxis; *2*) arrest of fully dependent tumors by restoration of the disturbed homeostasis; *3*) retardation of autonomous but hormone-responsive tumors by antagonistic hormones or hormone analogues; and *4*) target cell destruction by coupling hormones with cytotoxic chemicals or radioactive substances.

In closing, allow me to challenge some prevalent views: Is it essential to base progression of tumors on the assumption that the tumor cells must always be permanently altered? Observations on fully dependent neoplasms indicate the contrary. They show that at the onset many tumors are highly responsive to their physiologic regulators and that this responsiveness diminishes or vanishes with time. Concentration of research on much altered ancient transplantable tumors floods the literature with fictitious findings and diverts attention from "natural" neoplasms.

Is it essential for a virus to lurk behind all neoplastic modifications of nucleic acid? If this were so, it would require modifications of nucleic acid by both chemicals and radiations, i.e., mutations, to be due to a virus, always present in the environment or hidden inside the cell as a stowaway.

Is it essential to suppose that the process of viral carcinogenesis is linked with arrest of differentiation?

In all examples sketched here, we dealt with fully differentiated cells. In transforming them to cancer cells during exposure to subthreshold doses of carcinogens, we merely increased their numbers. Bringing cells to multiplication entails a greater chance of free entry of viruses and carcinogenic chemicals, as well as greater sensitivity to radiations.

I have chosen to speak on the role of hormones in carcinogenesis because they play a powerful role in initiation and maintenance of growth of tumors induced by either of the three classes of carcinogens: chemicals, radiations or viruses—and because they seem to offer many possibilities for control of certain tumors.

I am indebted to associates named for citing some of their work yet to be published, and to John J. Furth for editorial corrections.

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Introductory remarks

HAROLD E. HIMWICH

Thudichum Psychiatric Research Laboratory, Galesburg State Research Hospital, Galesburg, Illinois

THIS SYMPOSIUM IS THE FOURTH in a series of inter-society programs concerned with experimentally induced changes of behavior and has been prepared by a committee composed jointly of representatives of the American Physiological Society and the American Society of Pharmacology and Experimental Therapeutics. As can be seen from the second half of this program, the intellectual interests of the American Society of Biological Chemists are also included.

One of the impelling motives for this series of symposia is the rapid development of the psychoactive drugs. This group involves not only tranquilizers and antidepressants employed in the management of patients but also psychotomimetic drugs, used partly therapeutically, but chiefly for the experimental production of psychotic-like mental conditions. Many disciplines are involved, including pharmacology as well as physiology, for drugs are used in these experiments as tools to study physiological functions. Irrespective of the disciplines used in these studies, correlations are suggested between physiological changes in brain parameters and effects on behavior.

Drugs furnish excellent tools for the study of the brain and mind. Just as we prefer spontaneously behaving, freely moving animals in our analysis of brain functions to either anesthetized or immobilized animals, we also prefer the use of drugs to operative interference with the brain. The advantage of drugs over extirpations is obvious, for we can observe the subject, whether man or animal, before, during, and after drug action is over; in a word, we have a reversible rather than the irreversible condition produced surgically.

The three preceding symposia were concerned entirely with an analysis of animal behavior. For the present one, Doctors P. B. Dews and K. R. W. Unna, the two other members of the Bi-Society Committee, have chosen the subject, "Effects of Hallucinogenic Drugs in Man." It is not necessary to detail to this society the great importance of experiments performed on animals. By their use, it is possible to study changes in brain constituents by extirpating brain parts. Animals can also be used for the implantation of deep electrodes more readily than this can be accomplished in man. In many instances such experiments reveal significant differences from species to species. By the same token, it is frequently unwise to extrapolate results obtained from infrahuman species to man and this applies especially in the case of the central nervous system. It is, therefore, preferable to perform studies on man and obtain human observations directly. That is one reason why "man" was chosen for the material to be presented here.

For this Symposium, the effects of drugs on man is the common denominator for both societies responsible for the program. The earliest observations of the actions of drugs on man were made by clinicians, and indeed, they still make important contributions. Their work is most valuable in the discovery of the effect of drugs on human behavior, as well as individual variations from man to man. Furthermore, information acquired by clinical observation is of great value in predicting real life situations. On the other hand, they do not permit the quantitative evaluations which can be obtained from the application of experimental methods of psychology. These objective methods yield results of a different order from those obtained by clinicians in terms of greater precision as well as better reproducibility and therefore yield firmer ground for generalizations.

Such studies include the area of visual perception, the meaning obtained from the sensory process while the visual stimulation is present. In a word, the cognition or knowledge of what is seen. The first portion of this program is concerned with the methods of experimental psychology applied to the effect of drugs on the visual percept. The drugs used are psychotomimetic drugs capable of producing alterations in behavior, as well as visual illusions, in which the perceptions have a basis in the external world but the interpretation is faulty. With visual hallucinations, however, the perceptions are without objective reality. The first paper by Dr. A. M. Ostfeld and the discussion by Dr. W. T. Lhamon are

concerned with such studies. Among other results, it is shown that modifications in retinal functions are less important for the alterations in perception than are changes in brain function.

Dr. S. I. Szara presents quantitative evidence for the endogenous formation of psychotomimetic substances in the body. He administered tryptamine derivatives and observed their hydroxylation in the sixth position. The latter, he finds, has stronger psychotomimetic powers than its parent substance. Doctors G. G. W. Brune and G. R. Pscheidt note temporal correlations between the urinary excretion of indole derivatives and behavior. In fact, indole derivatives of serotonin as well as tryptamine may increase even before the onset of behavioral disturbances. As suggested by Dr. N. J. Giarman, the free amines, rather than the bound forms of the serotonin, are the active substances associated with the worsening of behavior. But it remains to be elucidated which one of the compounds studied is most relevant. Is it serotonin, tryptamine, or some other derivative of an indole compound, perhaps the ones suggested by Dr. Szara?

The conclusions of Dr. Szara, as well as those of Doctors Brune and Pscheidt, may be regarded as receiving support from the experiments of Dr. S. S. Kety and coworkers, for the latter observed intensifications of abnormal behavior when they administered tryptophan, the source of indole derivatives in the body. They also produced exacerbations of psychotic symptoms with methionine, which makes methyl groups available for combination with indole compounds. Just where LSD fits into the biochemical suggestions mentioned above is not clear, although LSD, in addition to the indole configuration, contains a methyl group attached to a nitrogen atom. Dr. Giarman has still another suggestion in regard to the mechanism of action of LSD, namely, an increase of bound serotonin. Although the biochemical papers differ in experimental details, it seems clear that they are complementary to each other in presenting different facets on the same problem. They should, therefore, stimulate further work on the psychotomimetic properties of the indole derivatives.

Effects of LSD 25 and JB 318 on tests of visual and perceptual functions in man

ADRIAN M. OSTFELD[1]

Department of Preventive Medicine, University of Illinois College of Medicine, Chicago, Illinois

IT HAS BEEN OUR GENERAL PURPOSE to bring together in this investigation, the techniques of psychology, pharmacology, and clinical physiology in order to study systematically the spectrum of effects of the drugs used, and, at the same time, to gain more understanding of the relationships between the specific response made by each subject and the specific personality of that subject.

The extraordinary complexity of the central nervous system effects of these hallucinogenic agents has made it necessary to employ a large number of diverse techniques in studying their effects. I should like to point out to you some of the techniques we have used and some of the effects we have noted. I shall not discuss the very large individual variability in responses to drugs, or the contribution of the subject to the drug-subject interaction, but will confine myself to dealing with some of the more uniform drug effects.

The first half of the presentation will deal with the effects of two hallucinogenic agents on visual function. This portion of the study is aimed at elucidating the part played by the retina and by other portions of the visual system in drug-induced hallucinations. Alex E. Krill, Public Health Service fellow in Ophthalmology, was associated with me in the design and execution of these studies.

In the second half of the presentation, I shall deal with the effects of two hallucinogens on certain aspects of mood, perception, and attention. My associate, Richard R. Rosman, an experimental clinical psychologist, was largely responsible for designing and carrying out these studies.

Careful clinicopathologic analysis has demonstrated that visual hallucinations in man can arise from any level of the visual pathways; i.e., retina to the cerebral cortex (31). Moreover, the site of a lesion in the visual pathways cannot be predicted by knowledge of the nature of the hallucination. Elaborate and formed hallucinations may be reported by patients with optic nerve disease, and simple unformed ones occur in patients with visual cortical lesions. The problem of defining the site of action of hallucinogenic drugs in man, therefore, requires a systematic assessment of drug effect on all accessible portions of the visual system.

Various workers have attempted to gain insight into this problem by studying the effect of hallucinogenic drugs on different levels of the visual pathways. Purpura (25, 26) found that smaller doses of lysergic acid diethylamide (LSD) (less than 60 μg/kg) facilitated cortical responses to photic stimulation in unanesthetized paralyzed cats but in larger doses depressed the cortical responses. He also found that the visual cortical responses to lateral geniculate body stimulation were facilitated by LSD. Evarts et al. (11) studied the effects of LSD (1 mg/kg) on synaptic transmission in the visual system of the cat. They observed a pronounced decrease in the amplitude of the geniculate postsynaptic response to optic nerve stimulation; while certain synapses—those of the retina and those between the lateral geniculate radiations and visual cortex cells—proved to be extremely resistant to any blocking effects of this drug. They also noted that large doses of LSD (2.5 mg/kg) caused a decrease in the amplitude of the optic nerve response to photic stimulation of the retina.

The electroretinogram (ERG) has been studied primarily in animals. (2–5, 10, 17). Apter and Pfeiffer (3, 5) gave 0.10 mg LSD to anesthetized cats and found that spontaneous retinal potentials occurred about 10 min later. At the same time spontaneous potentials from the optic nerve and large spikes from the visual cortex were recorded. If the optic nerve was sectioned while spontaneous potentials were recorded, the spikes in the occipital cortex and the potentials in the proximal stump of the optic nerve disappeared, whereas those in the distal stump of the optic nerve and retina persisted. Similar findings with mescaline, atropine, and strychnine were reported (2, 4). Evans, Jacobson and Gestring (10) studied the effects of LSD (50–75 mg/kg) on the ERG in the opossum and noted, in most instances, spike-like activity, which disappeared within 24 hr. Jacobson and Gestring (17) found that doses of LSD smaller than 25 mg/kg had no effect on the ERG of the cat but doses greater than 50 mg/kg sometimes produced spontaneous

[1] This work was supported by Public Health Service Grant MY-2302.

potentials. Only one ERG study with a human subject has been previously reported. This subject, studied by Jacobson, (17) received 50 μg of LSD, did not hallucinate, and no ERG changes were observed.

Visual threshold studies have been reported in animals (6) and man (7). Blough studied the effect of LSD on the visual threshold of the pigeon and found a substantial elevation (6). Carlson (7) studied the effects of LSD (50–100 μg i.v.) on the absolute visual threshold in man. He found that threshold values for the entire adaptation curve were raised a small but definite amount.

In the previously cited studies, LSD and mescaline, drugs with similar central and autonomic nervous system effects and chemical structures were the hallucinogens employed. In the present study, the two drugs used, LSD and JB 318, are somewhat similar in hallucinogenic properties, but differ in chemical structure, autonomic and somatomotor effects. LSD is an indole amine with adrenergic autonomic effects: JB 318, a piperidyl derivative with anticholinergic properties. The hallucinogenic effects of the two drugs overlap but differ in some respects. LSD is more likely to induce brightly colored, vivid, but simply structured hallucinations such as mosaics, rings, dots and clouds: after JB 318, images are more likely to be gray, white or black and include people, faces, landscapes, and animals.

Retinal function was studied with the ERG and the dark-adaptation curve. A total of 40 experiments were carried out on 19 medical students. Twelve were males, all were between 21 and 30 years, and all were trained subjects, having participated in drug studies previously. All had normal visual function and were in good health. Studies were single blind. Drugs were administered by the oral route with subjects in the fasting state.

Three kinds of drug experiments were carried out: *1)* Ten subjects on 13 occasions received large hallucinogenic doses of LSD or JB 318; i.e., 75–100 μg LSD or 7.5–12.5 mg JB 318. *2)* To determine whether retinal effects of these agents were dose dependent, five subjects on five occasions received smaller nonhallucinogenic doses; i.e., 25 μg LSD or 2.5–5 mg JB 318. *3)* Four subjects received one nonhallucinogenic analogue of LSD and JB 318. These analogues were 1 methyl-*d*-lysergic acid butanolamide tartrate (UML 491) and *n*-methyl-3-piperidyl diphenylacetate (JB 808). In the doses employed herein, these analogues exhibited no hallucinogenic properties, but they induced autonomic effects qualitatively similar to the hallucinogenic agents (1, 22). The rationale for employing the analogues was as follows: If retinal effects occurred with both hallucinogenic and nonhallucinogenic agents, they probably were not related to the hallucinogenic properties but to some other effect that each drug and its analogue have in common. On the other hand, if only the hallucinogenic drugs induced retinal effects, these effects are probably intimately related to the hallucinogenic properties.

Approximately half the subjects received a drug on the first trial and no drug at the second trial, thus serving as their own controls; the other half served as controls first and received drugs at a later trial. In all experiments, subjects reported to the laboratory, their pupils were dilated with cyclopentolate hydrochloride, and an initial ERG and dark adaptation curve were obtained. In the control trials, the studies were repeated after 1 hr. In the drug trials, repeat studies were made after the subjects reported hallucinations or illusions; or, if these did not occur, in 1 hr. Brief interviews were carried out at 5–10 min intervals to determine drug effects.

Dark adaptation measurements were made with the Goldmann-Weekers adaptometer. All subjects received one training session with this instrument several days before the experimental trials. Each subject was placed in complete darkness for 3 min and then exposed to a preadapting illumination of 2,400 lux for 7 min. The stimulus was presented in the form of light flashes 1 sec in duration, the dark interval also being 1 sec. The subject serially indicated by pressing a button, the least amount of light he perceived, and the presses were recorded on a revolving drum. A constant rate of change of stimulus intensity was maintained. Both the "on" and "off" thresholds were noted and the approximate true absolute threshold was calculated as an average of these values according to the method of Gunkel and Bornschein (13). Each subject was tested at standard intervals for 30 min.

The dark adaptation curve has a characteristic shape as shown in Fig. 1. The initial steep descent and the first plateau are related to cone function. The plateau is called the cone threshold. The next descent is called the rod-cone break, and the final plateau is called the rod threshold. The evaluation of the dark-adaptation curve was based on measurement of *1)* the times of the final cone threshold, rod-cone break and final rod threshold, *2)* the value in log units of illumination at the final cone threshold, and, *3)* the value in the same units of the rod threshold at 8, 10, 15, 20, 25 and 30 min. Measurements were made without knowledge of whether the records were "controls" or made during drug trials.

The ERG examination included an evaluation of scotopic single flash responses at four or more intensities and scotopic single flash responses at the lowest intensity with a blue filter. The subject was at rest on a bed, and the lamp, a Grass PS-1 photic stimulator, was centered 24 in. above the tested eye. The active electrode, a Burian-Allen contact lens, was placed on the cornea of the eye to be tested and the indifferent electrode on the bridge of the nose. Ground electrodes were usually placed on both ears. Recordings were made with the direct ink-writing Grass electroencephalograph.[2] The evaluation of the ERG included three measurements and the *a* and *b* wave amplitudes at each stimulus intensity. The *a* wave is the name applied to the initial negative deflection of the ERG, the *b* wave to the subsequent and usually

[2] Dr. Krill has recently made 40 ERG recordings simultaneously using the EEG and an oscilloscope and camera as recorders. He concluded that for amplitude measurements, with which we are concerned here, the two recording methods are comparable. For time measurements the EEG is less accurate.

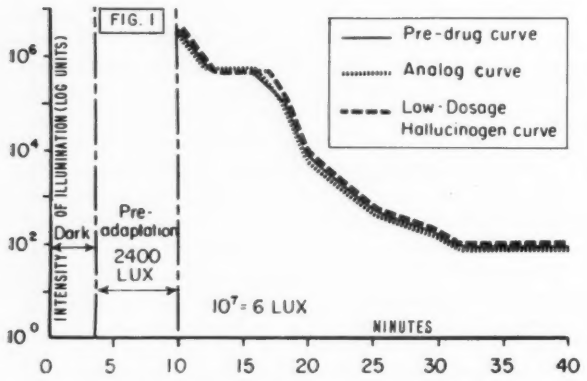

FIG. 1. Dark-adaptation curves for trials with nonhallucinogenic analogues and low-dosage hallucinogens. Values of each curve were plotted from averages of all trials.

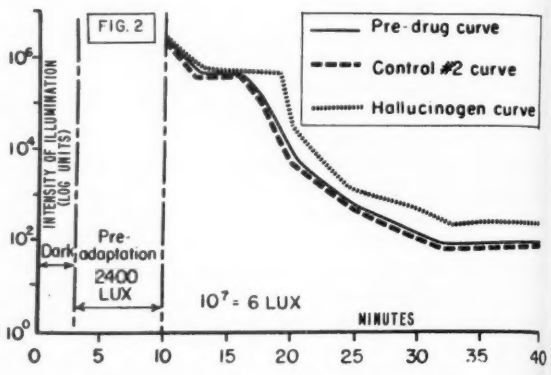

FIG. 2. Dark-adaptation curves for trials with hallucinogens and with no drug (control). Values of each curve were plotted from averages of all trials.

larger positive deflection. Discussion of the origin of these potentials is beyond the scope of this presentation.

Fifteen of the sixteen subjects who received the larger doses of LSD or JB 318 reported the occurrence of illusions and visual hallucinations. Six subjects who received the lowest doses of these drugs did not report hallucinations or illusions. Similarly, no visual effects were noted after the nonhallucinogenic analogues were administered.

Dark adaptation studies revealed essentially similar findings for control, nonhallucinogenic analogue and low dose hallucinogenic trials (Figs. 1 and 2). The average differences between the threshold values of the first curve (first control or predrug curve) and those of the second curve (second control or postdrug curve) were always less than 0.2 log unit—most often less than 0.1 log unit. All values of the second curve tended to be slightly lower than those of the first. The time values were almost the same for the first and second curves, the largest difference being about 1 min. The dark adaptation curves during the period when hallucinations were reported showed a different pattern (Fig. 2). All the rod threshold values of this curve were noticeably higher than the predrug curve and the rod-cone break usually occurred later. The average rod threshold difference between predrug and postdrug curves ranged from .4 to .7 log unit and the average rod-cone break was delayed about 3 min in the postdrug curves.

In the ERG measurements, the effect of the retest on *a* and *b* wave amplitudes was similar in control, nonhallucinogenic analogue and low dose trials. The *a* wave remained about the same or showed only a slight decrease in amplitude, whereas the *b* wave usually decreased in amplitude on repeat testing. On the other hand, records obtained while subjects were reporting hallucinations almost always revealed an increase in the *b* wave amplitude and very frequently the *a* wave amplitude. The average increase in amplitude for each subject for the *b* wave was from 27 to 41 μv, that for the *a* wave from 8 to 16 μv. The range of *b* wave changes included increases in amplitude of up to 100 μv. The changes in amplitude at four stimulus intensities are shown in one subject, who reported hallucinations after receiving 10 mg JB 318 (Fig. 3).

Statistical analysis was made on the changes in the ERG and in dark adaptation. In view of our still limited knowledge of the normal human ERG, no assumptions were made about the distribution of the data and the direction of the results was not predicted in advance. Therefore, a two-tailed nonparametric test, the Wilcoxon matched-pairs signed-ranks test, was employed (13).

There was no significant difference in any aspect of the data between the first and second control measurements, and between the predrug and postdrug measurements for the smaller doses of JB 318 and LSD and for the nonhallucinogenic analogues.

The analysis for the larger doses of LSD and JB 318 was done both for each drug separately and for the two drugs together. The only difference between the two analyses was in the significance of the *a* wave changes. Significant changes ($P < .01$) included: *1*) delay in the rod-cone break, *2*) elevation of the rod thresholds at each time period and, *3*) increase in the amplitude of the *b* waves at every intensity. The changes in the *a* wave amplitude were not uniformly significant. For the analysis involving both drugs together the changes in the *b* waves were significant at each intensity. The *a* wave changes were significant ($P < .05$) at intensities 1, 2 and 16 and not significant at intensities 4 and 8. When the effects of both drugs were evaluated separately, the *a* wave changes were not significant at any intensity; the *b* wave changes were uniformly significant. Since the *a* wave is usually more resistant to circulatory or drug effects, it is not surprising that the amplitude changes were smaller and less frequent than those of the *b* wave.

The results of the dark adaptation and ERG studies demonstrate significant changes in retinal function in association with hallucinations. The changes in visual threshold or dark adaptation were evident in the entire rod portion of the curve and indicated a mild impairment of rod function. These changes consisted of an ele-

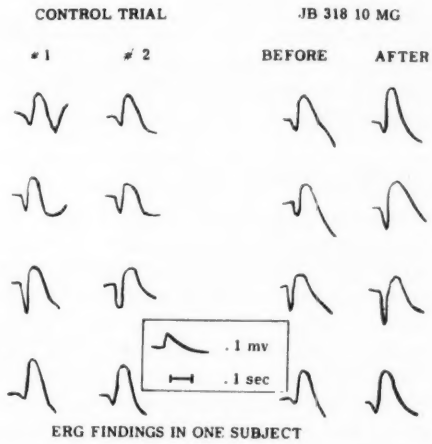

FIG. 3. ERG findings in one subject showing a control trial and a hallucinogen trial (JB 318). Note decrease in amplitudes in second column (control 2 record) and increase in amplitude in fourth column (postdrug record).

vation of the entire rod adaptation curve and a delay in the rod-cone break. The absence of change in the cone portion of the curve indicates a lack of measurable drug effect on cone function.

There is good reason to believe that the increase in the scotopic *b* waves induced by these drugs is related to a toxic or hypoxic effect. The *b* waves may increase in mild circulatory disturbance of the retina in man (14–16). Nakajima demonstrated that several compounds that inhibit retinal respiration and retinal glycolysis also cause an initial increase in the scotopic *b* wave amplitude (20, 21).

In summary of this part of the presentation, we believe we have demonstrated the following points:

1) LSD and JB 318 in hallucinogenic doses elevated the entire rod threshold and delayed the rod-cone break in the dark adaptation curve. They increased the amplitude of the scotopic *b* wave. These changes were exhibited while the subjects were reporting hallucinations.

2) No significant changes in retinal function were found in six subjects who received smaller nonhallucinogenic doses of LSD and JB 318, in four subjects receiving nonhallucinogenic analogues of these agents, and in 14 subjects participating in control trials.

3) The changes noted were interpreted as hypoxic or toxic retinal effects of the hallucinogens. These findings indicate that the hallucinogenic affect of these psychotomimetic agents may be related to concomitant change in retinal function.

Having established that retinal function is altered while hallucinations are being reported, we then asked: Are the drug-induced retinal changes causally related to the hallucinations? The next study was devised to try to answer the question. While the study is not complete, the answer is already emerging.

To date 17 totally blind persons have served as subjects. By totally blind, I mean without light perception or measurable retinal electrical activity. Four of these subjects have been blind from birth. They constitute a special case, and I shall not discuss them here. Of the remaining 13, 5 had had bilateral enucleations of the ocular globe. If, in properly controlled studies, these subjects "hallucinated" after receiving these drugs, then it is likely that neither the retina nor changes in its function are necessary for the occurrence of hallucinations.

We were taking a chance, scientifically, in carrying out these experiments. Little is known in man about transsynaptic degeneration of the visual system after retinal or optic nerve destruction. Isolated case reports support the notion that it does occur (8, 12, 24). Still less is known about the possibility of physiological atrophy of the central portions of unused sensory systems. These phenomena could have prevented our obtaining an answer to the question we asked. The absence of visual hallucinations after drugs in these subjects could mean merely that there were a physiologically or anatomically atrophied visual system incapable of responding to drugs in the same way as in normal subjects. A negative result would therefore have not been interpretable. At present, our results do seem capable of interpretation.

As implied earlier, the subjects all had had relatively normal vision for at least the first several years of life. Detailed medical histories were obtained from these patients. We intend to try to explore the relevance of number of years of blindness, rate of progression of blindness, and similar variables to the nature of drug effects. Detailed tape recorded interviews were made. We wanted to know if blind people "see" in their dreams, to know if they retained a visual memory of that part of their lives in which they could see, to learn if they could describe a scene to us out of visual memory if supplied with a few verbal cues, and whether or not they had spontaneous "visual" experiences resembling drug-induced ones in sighted persons.

We learned that most of the subjects in the group did "see" in dreams for from 5 to 10 years after the onset of blindness, but that this ability slowly disappeared thereafter. The capacity to remember things seen in earlier life had not disappeared in any of them. Spontaneous visual alterations were reported by 9 of the 13 patients. These consisted of light flashes; usually white, and colored or white spots. Only 4 of the 13 were able to describe in visual terms, an elaborate scene. The rest described standard scenes in terms of sounds, feels and smells. This ability to describe a scene in visual terms was limited to those with the most formal education and with the highest degree of verbal facility and general intelligence. The interrelationship between these various capacities and the nature of drug effects are being studied.

We were aware that our data would consist of verbal reports and, therefore, that the experimental design should be most rigorous. The study itself was "triple blind." The subjects did not know what drugs they were getting or the purpose of the study. As interviewer, we employed a medical student who was kept ignorant of the purpose of the study and the drugs to be used. He knew

almost nothing of psychopharmacology. He did not even know if drugs or placebos or both were being used. All information given to patients and all requests to be made were typed on cards and read to them so that every subject knew—insofar as we could determine—the same facts. All patient-interviewer interactions were tape recorded.

In these studies only LSD, 75 μg orally administered, or placebo was used. JB 318 is hard to take in hallucinogenic doses even for normal persons, so it was omitted. The subjects were randomly assigned to two treatment groups. One was to receive placebo first and then, a day later, LSD. The other group received drug first and then placebo.

Our results to date may be briefly summarized. Blind subjects reported significantly more "hallucinations" (unusual visual experiences) after LSD than after placebo. Uncommonly they reported elaborate and formed hallucinations. The visual experiences reported by blind subjects were qualitatively similar to those reported by sighted persons except that complex imagery occurred less often than might have been expected in normals. The blind subjects reported appreciably more auditory, tactile, olfactory, and gustatory hallucinations than normal persons usually do. Their visual "hallucinations" resembled the effects reported by Penfield after electrical stimulations of the occipital cortex (23). They consisted usually of spots or light flashes, often, but not always, colored. The complex scenes reported by our subjects bore no resemblance to those reported by Penfield's subjects after temporal lobe stimulation.

While we cannot be certain that the mechanism of these drug-induced effects in sighted and nonsighted persons is the same, we have drawn two inferences: *1*) A functioning retina is not a sine qua non for the hallucinogenic effects of LSD; *2*) the retinal changes induced by LSD are concomitant with but may, or may not, be casually related to the hallucinogenic effect. These conclusions are further supported by the fact that nonvisual hallucinations did occur and, therefore, other parts of the nervous system must have been affected by these drugs.

The second part of the discussion concerns itself with material more purely psychological.

In one sense, the material I shall present is taken out of context. The basic purpose of the work is to test certain hypotheses about the influence of some personality and perceptual variables on the effects of these drugs. I am presenting the work as if its purpose were to study the effects of these drugs on certain kinds of performance.

The subjects in these studies were, surprisingly enough, neither medical students, narcotic addicts, nor mental patients. They were electricians, cab drivers, and hospital orderlies—all young men—16 in number; half Negro and half White, carefully screened and, insofar as we could determine, free of significant mental or bodily illness.

In general, the experimental design followed these lines. After detailed psychological testing and evaluation, the men were randomly assigned to two groups. One received LSD first and JB 318 a week later. In the other, the order was reversed. Doses were for LSD, 75 μg, and for JB 318, 10 mg orally administered in cherry flavored solutions to prevent distinguishing the two. I shall discuss the effects of these agents on three psychological tests.

APPARENT HORIZON TEST

This test evaluates the subject's ability to estimate the height of the objective horizon. The objective horizon (OH) can be defined as the horizontal plane bisecting both eyes under normal forward gaze. The apparent horizon (AH) can be defined as that point or line in space which is perceived to be at eye level. The task of estimating the AH was presented to each subject twice on each experimental day: once when he reported for the experiment, and once during the period of maximum drug effect. Each administration consisted of three trials, or estimates, of the AH. The score for this test is the distance in centimeters between the AH as estimated by the subject and the OH as measured by the examiner; these errors may be in an upward (+) or a downward (−) direction.

Previous investigations have determined that estimates of the AH show considerable variation in several naturally occurring and experimental situations. Rosenblatt (27) reported that estimates of the AH were significantly higher for manic psychiatric patients than for depressed patients. Estimates made by normals fell between those made by the two patient groups. Wapner and Werner (30) reported that estimates of the AH were shifted to levels higher than base line estimates by subjects experiencing academic success, and to lower levels by subjects experiencing failure. Krus, Wapner, and Freeman (18) showed that estimates of the AH were shifted to levels higher than base line by subjects who had ingested iproniazid; there was a significant tendency for estimates to shift to levels lower than base line by subjects who had ingested reserpine.

These investigators accounted for these results by reference to the mood of the subject at the time of testing. Elevated, euphoric or manic mood states were associated with higher estimates and depressed states with lower estimates.

The purpose of this aspect of the experiment was to explore the effects of LSD and JB 318, and most particularly in their mood effects, upon estimation of the AH. These two drugs which have similar effects on many aspects of mental functioning have rather different effects upon mood: LSD frequently induces a giggly or happy condition; and JB 318 a tired, anxious, slightly depressed state.

To test the hypothesis that there was a difference between the several treatments, a treatment × subjects analysis of variance was performed on the data. This analysis indicated that there was no significant difference between the two control administrations; that estimates of the AH went up significantly following ingestion of LSD and down significantly following ingestion of JB

318, so that these two drugs were significantly different from each other, as well as from their respective controls, in their effect on the estimate of the AH. The average settings in centimeters were: LSD control −0.70, LSD experiment + 1.62, JB 318 control −0.62, and JB 318 experiment −1.62 ($P < .02$, two tail). A similar analysis was made to determine the effects of experimental order on the results. This analysis indicated that there was no significant difference between *control 1* and *control 2* or between experiment I and experiment II.

On the basis of these data we feel that the following inference is justified: Ingestion of LSD is followed by a significant increase in estimate of the AH, and ingestion of JB 318 is followed by a significant decrease in estimate of the AH.

CLYDE MOOD SCALE

This is a 133 item adjective check list (9). All the adjectives are descriptive of certain moods and cluster into six intercorrelated factors or mood scales. The scales were named empirically by Clyde. Each subject completed six mood scales, one immediately before each drug (I), one at the peak of drug effect (II), and one after drug effects were largely dissipated (III) (Table 1). The scores were analyzed by treatment × subjects analysis of variance. All P values specified are two tail.

The "depressed" scale was raised significantly by both drugs and to about the same extent ($P < .01$). The control scores did not differ. Both drugs raised the "jittery" scale significantly above its respective predrug levels ($P < .01$). LSD had a greater effect than JB 318 ($P < .02$). Controls did not differ. Both drugs reduced the "energetic" scale scores significantly below control levels ($P < .01$). JB 318's effect was greater ($P < .01$). There was no significant difference between control (predrug) scales. There was a nonsignificant tendency for LSD to raise the level of the "aggressive" scale. There was also a nonsignificant tendency for JB 318 to lower scores on the "aggressive" scale. Predrug scores on this scale were not significantly different. Both agents reduced scores on the "clearthinking" scale ($P < .01$).

A control group of eight subjects took six mood scales over the same time period and showed no significant changes in any of the scales on any administration.

TABLE 1. *Mood Scale Changes Associated with Ingestion of LSD and JB 318*

	LSD			JB 318		
	I	II	III	I	II	III
Depressed	74.8	89.4	82.0	73.3	90.7	75.6
Jittery	2.7	8.0	4.1	2.1	5.3	3.2
Energetic	21.8	18.7	18.4	21.9	15.1	18.2
Friendly	58.1	54.7	53.3	59.2	48.8	54.1
Clearthinking	17.6	13.9	16.9	18.3	13.3	16.8
Aggressive	15.9	18.3	15.6	15.7	14.3	15.3

Data are group mean raw scores.

The three scales on which there was a significant difference between LSD and JB 318 effects are *1*) the "jittery" scale; *2*) the "energetic" scale, and, *3*) the "friendly" scale.

These scales seem to be made up, respectively, of *1*) items relating to a poorly controlled excited or tense state, *2*) items relating to a well controlled tension or active state, and, *3*) items relating to investment in interpersonal relationships.

Ingestion of JB 318 seems to be followed by a reduction of active energy and a curtailment of investment in interpersonal aspects of living.

Ingestion of LSD seems to be followed by an increase in the amount of poorly controlled nervous tension or excitement.

We might speculate, then, that increases in the estimates of the AH following ingestion of LSD may be related in part to this heightened state of nervous tension—not necessarily to the happy or pleasant aspects of a euphoric state alone. Similarly, we might suggest that the decrease in AH estimates noted following ingestion of JB 318 may be related to a general reduction in outer-directed energy—the individual becomes somewhat less interested in the environment, and has less energy available for perceiving and coping with it. The latter pattern of energy change, often associated with depression as a clinical entity, may be as closely related to the change in estimate of the AH as the experienced mood quality of that (the depressed) state.

STROOP TEST

There are three parts or tasks in the Stroop test (29). In task 1, the subject is required to read as rapidly as possible 100 randomly arranged names of colors printed on a card. In task 2, he is required to name as rapidly as possible the color of 100 randomly arranged colored circles printed on a card. Task 3 is more complex. On the third card, the word RED may be printed in blue ink; the word GREEN in yellow ink. The subject is required to name the color of the 100 randomly arranged colored inks and not to read the printed words. In effect, as Rosman postulates, he is required to inhibit an habitual response—to read words—and facilitate a novel and conflicting one—to name colors.

The test was administered six times to each subject; twice during the preliminary evaluations, once immediately before each drug administration (predrug), and once again during the peak effect period of each drug (drug). The Stroop test data were analyzed by means of a type 1 analysis of variance, which may be regarded as a series of treatment × subjects analyses performed as several levels of a second variable (19).

The scores upon which this report is based are the times in seconds taken by the subjects to complete task 1 and task 3. Task 1 is thought of as making the simplest demand upon the subject, and task 3 is thought of as making the most complex demand upon the subject's mental apparatus.

The following findings relating to experimental order were pertinent: In task 1 the mean times of the two control administrations tended to decrease with practice, but these differences were not statistically significant. Furthermore, the mean times of the two drug administrations of task 1 did not differ significantly from one another. The mean times of the two drug administrations of task 1 were significantly higher than the mean times of the two predrug administrations ($P < .04$, two tail). In task 3 the mean times of the two control administrations tended to decrease with practice, but these differences were not statistically significant. Furthermore, the mean times of the two drug administrations of task 3 did not differ significantly from one another. The mean times of the two drug administrations of task 3 were significantly higher than the mean times of the two predrug administrations ($P < .01$, two tail).

The following findings relating to drugs were pertinent: The mean times of the pre-LSD administration of task 1 did not differ significantly from the pre-JB 318 administration. Moreover, the mean times of the two drug administrations, LSD and JB 318, did not differ significantly from one another. However, the mean times of the two drug administrations were significantly higher than the mean times of their associated control administrations ($P < .04$, two tail). The mean times of the pre-LSD administrations of task 3 did not differ significantly from those of the pre-JB 318 administration. Nor did the mean times of the two drug administrations differ significantly from one another. Yet, the mean times of the two drug administrations were significantly higher than the mean times of their associated control administrations ($P < .01$, two tail).

The means of the two tasks under control and drug conditions are presented in Table 2.

On the basis of these findings the following inferences seem to be justified: *1*) The drugs LSD and JB 318 increased the amount of time subjects took to complete task 1 and task 3 of the Stroop test, and the effects of these two drugs on these tasks did not differ significantly. *2*) The order in which the two drugs were given did not significantly affect their influence upon the Stroop test.

Extensive control studies indicated that pupil dilatation of the degree produced by JB 318 or LSD had no effect on Stroop test performance.

Detailed interpretation of these data must await further studies with the Stroop test. At the present time, we would tend to think about these results as follows:

1) The drugs probably impair performance on the Stroop test by reducing the efficiency of mental operations at a rather basic level, involving attentional processes, and possibly also by reducing the efficiency of the processes underlying motor expression. We base this inference upon the fact that ingestion of both drugs is followed by significantly increased times taken to perform task 1 of the Stroop, a task that requires the subject only to repeat performance of an already overlearned repetitive task. We believe it likely that the drugs must effect the basic mental operations to significantly alter performance on this type of a task.

2) The drugs probably also impair performance on the Stroop test by interfering with more complex mental functions, such as those underlying concentration and maintenance of set. We base this inference upon the observation that ingestion of both drugs is followed by an increase in time to perform task 3 much greater than the increase associated with task 1. We postulate that this added time increase is related to interference by the drug with the mental processes required for performance of the third task.

3) Although these data do not bear directly on this point, we ought to mention the possibility that the effect of these drugs upon the Stroop test may be due in part to an effect upon the quality of the motivation of the subject. Either a reduction of the energy with which he attacks this task, or a reduction of the desire to do well on it, might be associated with increased time to complete the task. The examination of this possibility is required by observations made by us and by other experimenters of a lethargy and inertia—a "not feeling like doing anything" state—sometimes following ingestion of LSD and JB 318; and of a mildly euphoric, easily distracted—an "aw doc, I don't wanna" state—sometimes following ingestion of LSD.

These data suggest several lines of interesting follow-up investigation. We are at present engaged in some work which we hope will tell us something about the perceptual-mental processes underlying performance of the three tasks of the test. Briefly, this work involves tachistoscopic exposure to subjects of the Stroop stimuli under all possible combinations and with several sets of direction. We will be interested, *a*) in the response times and recognition times under the various conditions, and, *b*) to the subject's description of the tasks as he perceived them and his analysis of his approach to meeting their various requirements.

In summary, we believe that our results indicate an extraordinary complexity of the effects of these drugs. They alter the function of peripheral sensory organs and affect the central sensory systems as well. They change mood and perception. They alter operation of basic mental functions, such as alertness and attention and, therefore, impair performance on simple mental tasks. It is the hope of those of us who work in this area that precise knowledge of the behavioral effects of these agents, coupled with exact knowledge of their mechanism of action, will lead to an understanding of the functioning of the normal central nervous system.

TABLE 2. *Stroop Task Times in Seconds Under Certain Conditions*

	Pre-LSD	LSD	Pre-JB 318	JB 318
Task 1	35.81	43.50	36.00	41.81
Task 3	87.18	106.06	86.68	103.62

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Discussion of Paper by Dr. Ostfeld

WILLIAM T. LHAMON

Department of Psychiatry, Baylor University, Houston, Texas

DR. OSTFELD'S RESULTS, in brief summary, are as follows: *1*) He has shown that hallucinatory doses (75–100 mg LSD and 7.5–12.5 mg JB 318) produced prolongation of the rod-cone break in the dark adaptation curve of the electroretinogram. The *b* wave of the ERG was increased by both drugs. Both experiments were interpreted as indicating hypoxic or toxic effects on the retina. *2*) He has shown that hallucinatory dose of LSD on 13 blind persons, who had been able to see at one time, produced more visual "hallucinatory" activity than placebo. *3*) On a test of "apparent horizon" it was found that LSD raised and JB 318 lowered the AH. *4*) Using an adjective check list, it was shown that both drugs usually depressed mood; both produced "jitter," with LSD more; both "lowered energy," with JB 318 more; and both interfered equally with clarity of thought. *5*) On the Stroop test of performance speed on a visual discrimination task it was shown that both JB 318 and LSD slowed tasks 1 and 3 of the Stroop test, and task 3 more.

The dark adaptation and ERG results are interesting and seem carefully designed and worked out. I would expect these data will surely soon be corroborated. The comparison study with blind people is a relief to have. It has always been most difficult to believe that the complex percepts produced by LSD and other hallucinogens can result only from alterations in the retina. Complex forms, familiar sights, and persons, both in illusion and in hallucinations, clearly can issue from experience with these drugs; at times these experiences seem perfectly real to the subject. It is very hard to consider retinal pathology alone responsible. Dr. Ostfeld's positive findings with blind persons indicate a central process.

This central process of perception, of abstraction, and of conceptualization must, I think, be a process of comparison, in which a received pattern from the sense apparatus is compared for sufficient congruity with a previously received pattern selected from memory. If the current and earlier patterns are sufficiently alike, a

meaningful sensation or perception will be experienced. Surely, a process with memory searching, with comparing for some degree of congruity, with a specifying of the degree of congruity required, and with making sure of the incoming sensation, is a complex process and intuitively seems most unlikely to occur only at the peripheral receptor.

Tanner (6) has recently outlined a model of this sort, in which memory is introduced as a modifying factor in an otherwise purely sensory system. From the point of view of someone interested in psychopathology, and the unfortunate results of unhappy earlier experience as it influences present behavior, a psychophysics which includes what the subject brings to the situation is more interesting than a psychophysics focused on sensory thresholds and metering type discrimination. In my opinion the former type of study, in which historical experience is considered, will prove more sensitive than the latter in detecting differences between drug action or illness on behavior.

Thus, in a study of LSD on the time sense (1), a method of absolute judgment was used and showed an effect of increased variation of judgment in controlled studies of doses of 4, 7 and 12 μg. Furthermore, this result was different from results with schizophrenic patients (5); in terms of absolute judgments of seconds, schizophrenia is qualitatively and quantitatively different from LSD toxicity. Schizophrenic patients showed a shortened private second; with LSD subjects this result was not obtained. On the other hand, LSD subjects showed a variation of judgments greater than healthy controls or patients. The situation was described, "as an intermittent dropping out of what a second is" but with no failure to sense the audible stimuli. The method of absolute judgment of time has been sensitive to a number of other agents or conditions. Amphetamine (2), hyperthyroidism (8), and raised bodily temperature (8), shorten the private second (with overestimation of presented durations), while secobarbital and boredom (2) lengthened it, resulting in underestimation of presented durations. Whether these changes represent changes in the memories for a second, in the sensory detection of stimulus, or in comparing process, we do not know.

A number of other factors appear to influence absolute judgments. Thus, the first stimulus in a sequence acts as an "anchor," so that the resulting judgments are pulled towards the "anchor" (4). Acute schizophrenic patients (7) show a rapid decay of anchor effects and seem "isolated in the present," insofar as their response to relatively recent remote experience goes. This sort of study could well be applied to drug responses and I would like to know whether Dr. Ostfeld has run into any such effects.

Apparent horizon changes are interesting. I cannot suggest an explanation for this finding that improves on the one presented.

There is much mystery surrounding the different modes of perception. Thus, we find more visual than audible "time" is required for one apparent second (3).

Stroop results reported by Dr. Ostfeld are interesting. I agree that task 3 of the test is more difficult than task 1, because of the habit conflict forced on the subject. I also feel that task 1, which involves reading words, is more difficult than task 2, which requires only the matching of response to colored ink. I would like to know how LSD and JB 318 subjects did on task 2.

The author has presented sensory correlates of drug response in terms of dark adaptation and ERG. He also has presented some fascinating perception experiments. My reaction to both is one of interest, with a belief that the perceptual studies, despite all their complexity, have a depth and a sensitivity lacking in purely sensory studies. The organism's contribution of learned and experienced event from memory modify sensations and the responses thereto, increase the sensitivity of the experiments, and challenge the ingenuity of the experimenter.

Hallucinogenic effects and metabolism of tryptamine derivatives in man

STEPHEN SZARA

Clinical Neuropharmacology Research Center, National Institute of Mental Health, Saint Elizabeths Hospital, Washington, D. C.

I BECAME INTERESTED IN THE POSSIBILITY of hallucinogenic action of alkylated tryptamine derivatives in 1955, when I read about the chemical analysis of a snuff powder prepared by Haitian natives from Piptadenia Peregrina seeds which they used in religious ceremonies to produce mystical states of mind which enabled them to communicate with their gods. The chemical analysis by Fish, Horning and Johnson revealed the presence of bufotenin and a small amount of N,N-dimethyltryptamine (DMT) (7).

The hallucinogenic action of bufotenin was reported by Fabing. It was suggested that the psychological action of the snuff powder (called Cohoba) was primarily due to its bufotenin content (6).[1] In 1955, there was no information available whether DMT had similar psychotropic activity.

A relatively simple synthesis, published by Speeter and Anthony, made it possible for us to synthetize DMT and a few higher homologues (14). After toxicity studies in animals, we started to study the psychotropic activity of DMT, first on normal volunteers, and then on patients. The results were reported at the time (4, 9, 13, 15, 16) and were confirmed by other independent investigators (3, 19); therefore, I will restrict myself to mentioning only a few pertinent features.

a) The first and perhaps most striking feature of the effect of DMT is the extremely rapid onset of the symptoms (2–5 min after the intramuscular injection of 1 mg/kg in normal volunteers) and the very short duration of action (40–50 min) in contrast to the 4–10 hr duration of similar symptoms produced by LSD_{25}, or mescaline. This clinical feature pointed to the probability of a very rapid metabolism of the drug and to a more direct action on the CNS.

b) Another important observation was that there was a very striking individual variation in the reaction to the drug, both in the intensity and the symptomatology, after giving it to each subject in the same dose.

The variation in the intensity seems to be very closely related to the individual variation in the metabolism of the drug. This will be discussed later. First, however, I would like to say a few words about the variation in the symptomatology of the drug effect in general.

It is well known that the phenomenology of the psychotropic drug effect, and I am talking here not only about DMT, but DET,[2] mescaline, LSD, and psilocybin as well, depends on many factors.

One group of factors lies in the personality of the subject. These personality variables were comprehensively reviewed recently by Lindemann and Felsinger (10). Different individuals, even with the same mental condition, may react differently to any one of the drugs. These differences not only show themselves in the superficial structure of the changes produced, but reach down deeper into the personality and determine the levels which these effects may reach.

The other group of factors which influence the picture are situational ones. The drug effect is different when it is given in a psychotherapeutic setting (1, 5), in a dark room with maximal sensory deprivation (12), in a pleasant living room without any special "directing" activity (8), or during a religious ceremony.

It is interesting to note that in a psychotherapeutic situation, or sometimes without any structuring of the experiment, these drugs unlock the doors to memory, past experiences—and, it has even been suggested by Abramson (2) to the inborn images of the unconscious. On many occasions, the experience has been described to go even deeper. Defying the conventional categories of space and time, the subjects take long journeys into very vivid, hallucinatory experiences, and become aware of deep philosophical, transcendental, and mystical problems with concomitant deep emotional reactions. Sometimes it seems to me that "mysticomimetic" would be a much more appropriate designation for these compounds than the widely-used, but unjustified, name "psychosomimetic."

But let us return to more earthy realities, to the problem of the relationship between the intensity of the

[1] The hallucinogenic activity of bufotenin, however, has recently been questioned by Turner (19), and independently by H. Isbell (personal communication).

[2] N,N-diethyltryptamine.

drug reaction and the metabolism of the drug, particularly DMT, DET, and α-MT.

In Fig. 1 it can be seen that DMT is metabolized in two main directions. One direction is dealkylation and oxidative deamination, which leads to the formation of indolylacetic acid (3-IAA). The other way is 6-hydroxylation, followed mainly by conjugation and to a slight extent dealkylation and oxidative deamination leading to 6-HIAA. The metabolic route for DET is essentially the same, leading to the excretion of 3-IAA and 6-HDET glucuronide as the end products of the two major pathways.

It is interesting to note that one of these enzymatic steps, namely, the 6-hydroxylation, increases rather than decreases the psychotropic activity of the parent compound. In a series of animal experiments with Dr. Hearst, we have shown that 6-HDET has a significantly lower behavioral threshold in rats, pigeons, and monkeys in a variety of experimental situations using operant conditioning techniques (18). Since this is a unique feature among the various known hallucinogenic drugs, and since it has very interesting implications due to the close similarity in chemical structure to normally occurring compounds like tryptamine and serotonin, we paid extra attention to the enzymatic process of 6-hydroxylation. The results of these enzymatic studies were presented in a separate paper at an intersociety neuropharmacology session of this meeting (17).

In animal experiments we learned that the individual variation in threshold doses of DET is very closely related to the individual variation in the 6-hydroxylation of the drug (18). We suspected that the same metabolic factor, the rate of 6-hydroxylation, is responsible, too, for the individual differences in the intensity of the psychological reactions to DET in man. Therefore, we investigated the correlations between the intensity of the psychological reaction to this drug and the metabolism, especially 6-hydroxylation.

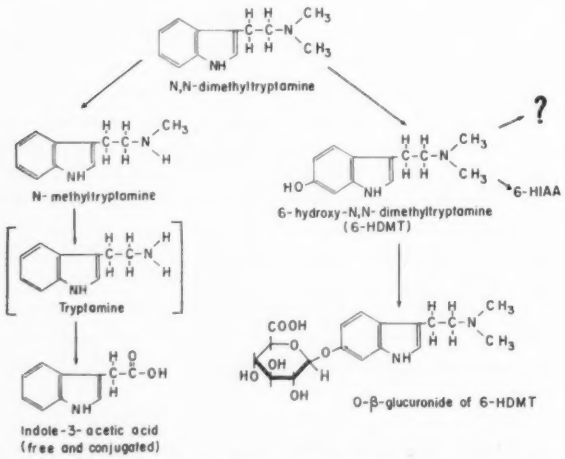

FIG. 1. Metabolic pathways of N,N-dimethyltryptamine in mammals.

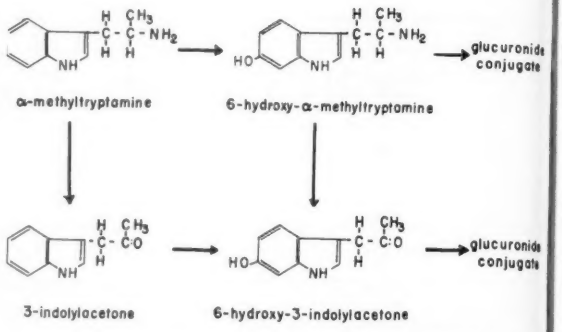

FIG. 2. Metabolic pathways of α-methyltryptamine in rats.

The DET was administered in the same dose (1 mg/kg) to ten normal volunteers, scoring the subjective and objective symptoms, and, at the same time, collecting the urine for quantitative determination of the excreted 6-HDET as a measure of the 6-hydroxylating ability of the individual organism.

The results, which are to be presented at the Third World Congress of Psychiatry in Montreal, confirmed our hypothesis derived from the animal studies, namely that subjects excreting more 6-HDET in the urine showed much more intense reaction to the drug than those excreting less 6-HDET. This result points again to the importance of 6-hydroxylation in producing the psychological effects of a hallucinogenic drug in man and invites speculation on whether or not the same reaction plays a role in producing psychotic 6-hydroxy derivatives from normally occurring indole derivatives, like tryptamine.

One would be tempted to assume that under specific circumstances, as in those experimental situations which are described in this symposium by Dr. Kety, or in the experiments of Drs. Brune and Pscheidt, when an increase in the concentration of tissue tryptamine occurs, there is a chance that a portion of tryptamine could be hydroxylated to form 6-hydroxy-tryptamine (6-HT). But 6-HT, having a lipid solubility similar to 5-HT, probably does not cross the blood-brain barrier readily, and one would not expect that 6-HT could produce CNS symptoms.

The situation, however, is not quite so unpromising. There is another compound which might give us some information in this matter. Another simple tryptamine derivative, α-methyltryptamine (α-MT), produces LSD-like symptoms which develop more slowly and last longer than the symptoms of DMT or DET intoxication and are slightly different as well (11). We found that α-MT is metabolized according to the following scheme (Fig. 2).

As will be seen, α-MT can be hydroxylated to form 6-HO-α-MT, then deaminated to 6-hydroxy-indolylacetone, or deamination occurs first, followed by 6-hydroxylation, to form the same compound. We found that

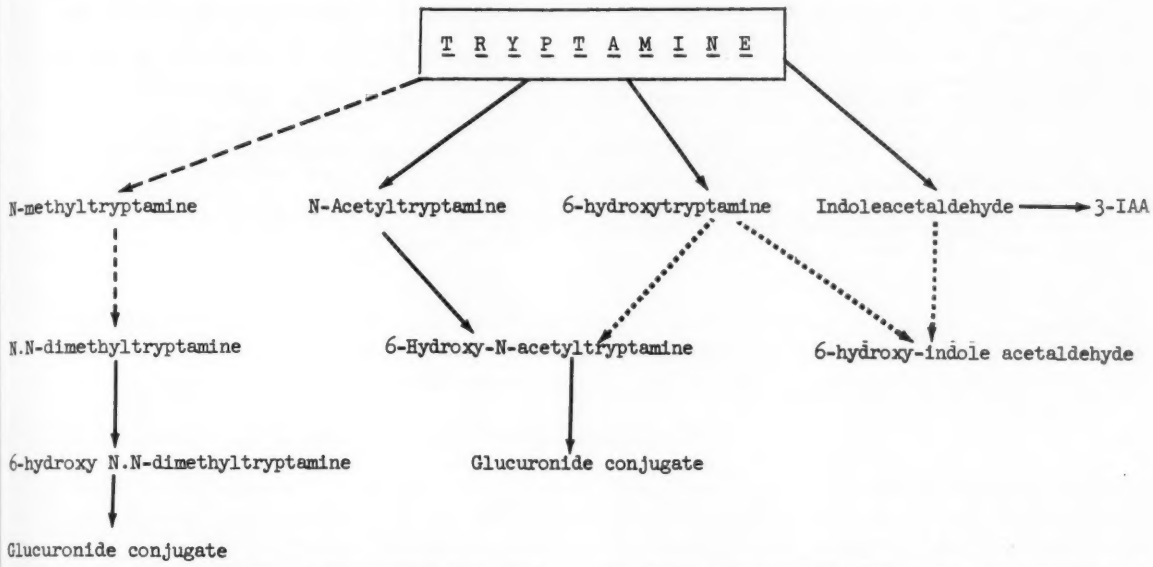

FIG. 3. Possible alternate pathways for tryptamine in mammals. *Solid arrows* represent paths demonstrated both in vitro and in vivo. *Broken arrows* represent paths shown in vitro only. *Dotted arrows* represent hypothetical pathways.

both pathways occur in the rat in vivo, and the hydroxy derivatives are excreted as glucuronides. In man, we found paper chromatographic evidence in the urine of 6-hydroxylation of α-MT. Since indolylacetone is found to be ineffective in producing behavioral changes in animals, we may hypothesize that 6-H-α-MT plays a role in affecting the behavior of animals and producing CNS effects in man. How is this accomplished by a compound which has lipid solubility similar to 5-HT, and therefore, little chance of getting into the brain? We don't know. The permeability of the blood-brain barrier is not uniform through the brain. Perhaps the metabolite affects only those portions which can be easily penetrated; the hypothalamus, and pineal gland area, etc.

Returning to our original problem, tryptamine might also be transformed into other more lipid soluble compounds, which would be more easily 6-hydroxylated and would penetrate the blood-brain barrier.

Three possibilities come readily to mind. One is N-methylation to form N-methyl tryptamine, or even DMT. This possibility seems feasible when we consider the interesting results obtained by Dr. Kety and his group in methionine and tryptophane loading experiments, and Dr. Axelrod's recent findings about the occurrence of an enzyme in mammalian organisms which actually can perform this reaction (J. Axelrod, personal communication). It seems that the whole enzymatic apparatus exists in mammals which can produce tryptamine from tryptophane, DMT from tryptamine, and 6-HDMT from DMT.

The second possibility for transforming tryptamine into a more lipid soluble compound before 6-hydroxylation is acetylation to form acetyl tryptamine (H. Weissbach, and J. Axelrod, personal communication). For the occurrence of this pathway we have preliminary evidence in animals. If we inject tryptamine intraperitoneally into rats or rabbits pretreated with Marsilid, we find 6-HT, 6-HO-acetyltryptamine and their glucuronide in the urine.

The third possibility emerges from studies reported by A. J. Friedhoff and M. Goldstein at the Conference on Some Biological Aspects of Schizophrenic Behavior in New York on April 6, 1961 (organized by the New York Academy of Sciences, and to be published in the Annals of The New York Academy of Sciences). They presented evidence that the intermediate aldehyde formed from mescaline by deamination is a strong candidate for an active metabolite in mescaline action. This finding suggests that we might consider the aldehyde's intermediates in other amines, like tryptamine or 6-HT as psychotropically active metabolites.

Whether these reactions are involved in Dr. Kety's findings with tryptophane and methionine loading experiments, and in the very interesting observations of Drs. Brune and Pscheidt in their reserpine and isocarboxazide combination, which is reported here today, remains to be explored.

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Correlations between behavior and urinary excretion of indole amines and catecholamines in schizophrenic patients as affected by drugs

GUENTER G. BRUNE AND GORDON R. PSCHEIDT

Thudichum Psychiatric Research Laboratory, Galesburg State Research Hospital, Galesburg, Illinois

SINCE THE INTRODUCTION OF PSYCHOTROPIC drugs, such as tranquilizers and antidepressants, life has improved for psychotic patients in our mental hospitals. At the same time, psychotropic drugs are excellent tools in research for obtaining new information on brain function and, perhaps, on underlying chemical factors associated with mental disease. Today, it is well established that some of the most important psychotropic agents exert effects on indole metabolism.

Reserpine, an alkaloid of Rauwolfia serpentina, releases bound serotonin (3, 21, 22). Because only those Rauwolfia alkaloids which are able to release serotonin in the brain exhibit psychotropic properties, it was concluded that there may be a relationship between the pharmacological action of the drug and serotonin metabolism (5). Recent investigations indicate that the phenothiazines, such as the tranquilizer chlorpromazine (2, 9) and the antidepressant drug imipramine (8, 18), may affect serotonin metabolism. Monoamine oxidase inhibitors are used as antidepressants in the treatment of melancholia and depressed psychotic patients. These drugs interfere with indole metabolism by blocking the conversion of the amines to their respective acids, and, thus, elevating the amine level in the brain (28). Such drug-induced metabolic changes apply not only to indoles, but also to catecholamines (4, 7, 15). Thus, both types of amines must be taken into consideration in interpreting the psychopharmacological effects.

Further evidence that the action of psychoactive substances may be connected with indole and catecholamine metabolism stems from studies with hallucinogens which produce psychosis-like states in man. Such a drug, chemically related to the catechols, is mescaline. LSD 25 is an indole derivative and one of the best-studied hallucinogens. Recently it has been shown that psilocybin, bufotenin, and N,N-diethyl-6-hydroxytryptamine (11, 14, 26) are potent hallucinogens. Chemically these compounds are characterized by the substitution of the amino hydrogens with either two methyl or two ethyl radicals and by hydroxylation of the indole nucleus in either the 4, 5, or 6 position. These psychotomimetic agents are discussed in more detail by Dr. Ostfeld and Dr. Szara.

The behavioral alterations produced by all these drugs both in animals and man stimulated a reinvestigation of indole metabolism in mental patients. In the past few years defects in indole metabolism were discovered in phenylketonuria (1) and Hartnup disease (16). Studies on schizophrenic patients did not yield conclusive evidence of an altered indole metabolism, either from quantitative or qualitative aspects, although results were reported suggesting a metabolic aberration (12, 17, 19, 20, 23, 24). Most of the results, however, are based on qualitative or semiquantitative tests, thus interpretation becomes difficult. Determination of urinary 5-hydroxyindoleacetic acid in schizophrenic patients did not reveal significant changes in comparison with normal subjects beyond a day to day variability (12, 13).

While investigating the effects of therapeutic doses of reserpine on the urinary excretion of tryptamine in schizophrenic patients, we observed abnormally high levels of that indole amine during a flare-up of the psychosis. This observation prompted a study on a greater number of schizophrenic patients.

METHODS

Among the requirements in the selection of the patients were good physical health and absence of feeding problems.

In longitudinal studies, the daily urinary excretion of the indole derivatives: tryptamine (25), total 3-indoleacetic acid (29), and 5-hydroxyindoleacetic acid (27), as well as of the catecholamines noradrenaline and adrenaline (10), were determined by quantitative chemical assay.

We attempted to determine whether or not there was a correlation between the biochemical findings and the

TABLE 1. *Scale of Psychotic Activity*

Degrees of Psychosis	Symptoms
A. Apparently inactive	Good hospital adjustment. Psychomotor activity adequate. Thinking coherent. No apparent delusions or hallucinations. Flat affect.
B. Slightly active	Fair hospital adjustment. Psychomotor activity increased or decreased. Thinking usually coherent. No apparent delusions or hallucinations. Flat affect.
C. Moderately active	Only fair or impaired hospital adjustment. Psychomotor activity abnormally increased or decreased. Thinking incoherent. Hallucinations and/or delusions present. Flat affect.
D. Active	Impaired hospital adjustment. Psychomotor activity abnormally increased or decreased. Thinking incoherent. Hallucinations and/or delusions more pronounced than in C. Flat affect.
E. Markedly active	Symptoms as under D to an exaggerated degree. Study could not be extended over 5 days because patients had to be given high doses of tranquilizers.

clinical observations. Based on daily psychiatric interviews and observations of the behavior of the patients on the ward throughout the day, the psychotic activity was scored in five different degrees, with particular respect to the categories: hospital adjustment, affect, psychomotor activity, thinking, delusions, and hallucinations (Table 1).

The patients were studied during placebo administration and while under treatment with therapeutic doses of psychotropic drugs in order to ascertain whether or not drug-induced alterations of psychotic activity were accompanied by characteristic excretion patterns of the tested indoles and catechols similar to those seen during the spontaneously occurring variations in the clinical symptomatology. We chose reserpine,[1] which does not interfere with the chemical assay, and, as known from clinical experience, can exert biphasic actions on the behavior of the patients. Especially in the initial period of administration, reserpine can induce a flare-up of the psychosis before its therapeutic effects are established. Biphasic effects on behavior can also be evoked by monoamine oxidase inhibitors. The monoamine oxidase inhibitor isocarboxazid[2] was administered separately and together with reserpine. The results obtained during that drug period, however, will not be considered here in connection with indole metabolism because during this time urinary tryptamine was elevated and that of 5-hydroxyindoleacetic acid was reduced in consequence of monoamine oxidase inhibition. Thus, this report includes four medication periods. Two of these (placebo 1 and reserpine 1) took place before, and the other two (reserpine 2 and placebo 2), after the combination isocarboxazid-reserpine had been administered.

Before the study and throughout its entire duration, the patients received a constant protein diet of 100 g protein per day, the actual intake being recorded daily. The 5-day averages of urinary indoles were calculated on a basis of 100 g protein per day. The results will be presented in the following sequence; including the correlation between behavior with the metabolism of: *1*) tryptamine, *2*) serotonin, and *3*) catechols.

RESULTS

1) *Tryptamine metabolism.* This part of the investigation was performed on 20 male schizophrenic patients, who were observed for an average of 42 days, including the four above-mentioned medication periods. All patients were studied during placebo 1; 17 patients during reserpine 1; 7 during reserpine 2; and 13 during placebo 2. A total of 835 24-hr urine specimens were analyzed. The range of normal for tryptamine was reported by Sjoerdsma, Oates, Zaltman, and Udenfriend to be between 36 and 120 μg/day (25). Weissbach and colleagues found for total 3-indoleacetic acid a normal range between 5.2 and 13.8 mg/day (29). A summary of all our data shows that the average tryptamine excretion was within normal limits (118 μg/day), while that of total 3-indoleacetic acid was elevated (18.1 mg/day). The average excretion of both indole derivatives during reserpine 1 and reserpine 2 did not differ significantly from that of placebo 1 ($P > 0.05$). Tryptanine excretion was significantly higher during placebo 2 than during placebo 1 ($P < 0.05$; $P > 0.01$). There was no significant increase for total 3-indoleacetic acid during the second placebo period, if compared with the first placebo period ($P > 0.05$). A wide range of variation was found in each group.

If urinary excretions of tryptamine and total 3-indoleacetic acid are correlated to the degree of psychotic activity (Table 2), it becomes apparent that the increases in the activity of the psychosis are paralleled by rises in the daily urinary excretion of tryptamine and its metabolic product 3-indoleacetic acid. At the 1% level, urinary excretion of tryptamine is significantly higher during the various degrees of increasing psychotic activity, B, C, D, and E, than when the psychosis was apparently inactive. The excretion of total 3-indoleacetic acid departs significantly from A during C, D, and E ($P < 0.01$), but the rise in B is not significant ($P > 0.05$).

As shown by the range of variations, there are individual differences. The lowest values for tryptamine during C, D, and E were excreted by the same patient whose urine volume was also very small. But this same patient eliminated large amounts of 5-hydroxyindole-

[1] Reserpine (Serpasil, Ciba), 4 mg/day, orally, dissolved in water.

[2] Isocarboxazid (Marplan, Hoffmann-LaRoche), 30 mg/day dissolved in water.

acetic acid. The values for group E may not be quite reliable, because of the small number of patients and days of investigation.

2) Serotonin metabolism. 5-Hydroxyindoleacetic acid excretion was studied on 17 male schizophrenic patients during placebo and reserpine periods. A summary of the results disclosed patterns similar to those reported for tryptamine and total 3-indoleacetic acid. A total of 685 24-hr urine specimens were analyzed, the average daily excretion was near the upper normal borderline, 7.0 mg/day (13). Again, reserpine had no effect on the urinary excretion of the serotonin metabolite when all data were considered (placebo 1 compared to reserpine 1, reserpine 2, placebo 2; $P > 0.05$). If, however, the degree of psychotic activity is included as shown in Table 3, increases of psychotic activity are paralleled by rises in the urinary excretion of the serotonin metabolite. The rise into abnormal range, however, is not as pronounced as was observed for tryptamine and total 3-indoleacetic acid, except in degree E. Again wide ranges of variation were found. The lowest values during B, C, D were excreted by a patient who, at the same time, eliminated abnormally high amounts of tryptamine and total indole-3-acetic acid.

TABLE 2. *Urinary Tryptamine and Total Indole-3-acetic Acid as Related to the Degree of Psychotic Activity*

Degree of Psychotic Activity	Days (No. of Patients)	μg Tryptamine/day		Mg Total Indole-3-acetic Acid/day	
		Average	Range	Average	Range
A. Apparently inactive	410 (17)	91.6	137 39	16.3	33.5 6.9
B. Slightly active	270 (12)	124.9*	167 58	17.3	45.2 6.3
C. Moderately active	105 (7)	151.2*	282 66	21.2*	49.0 12.1
D. Active	40 (6)	202.8*	292 153	28.8*	49.4 14.5
E. Markedly active	10 (2)	382.5*	585 180	43.1*	54.0 32.3

Ranges are represented by the highest and lowest 5 day averages observed in the single groups. * Probability of difference from degree A, $P < 0.01$.

TABLE 3. *Urinary 5-Hydroxyindoleacetic Acid as Related to Degree of Psychotic Activity*

Degree of Psychotic Activity	No. of Patients	Days	mg 5HIAA/day	Range in mg 5HIAA
A. Not apparently active	14	285	5.9	9.3 4.1
B. Slightly active	10	245	6.2	10.0 3.3
C. Moderately active	6	105	9.8*	14.1 6.0
D. Active	6	40	8.8*	15.3 3.9
E. Markedly active	2	10	17.8*	17.9 17.6

Ranges are represented by the lowest and highest 5 day averages observed in the single groups. * Probability of difference from degree A, $P < 0.01$.

3) Catechol metabolism. Urinary catecholamine determination was carried out in 12 schizophrenic patients. In some cases, daily samples were analyzed while in others pooled 5-day samples were used. In all, a total of 578 separate determinations were performed. These include the drug periods in which isocarboxazid was administered. During placebo periods a wide range of values were obtained. Most of the values were within the normal limits of von Euler and Floding (10). In this group of patients, we did not observe an activation of the psychosis while the patients were under drug treatment, whether on reserpine or reserpine-isocarboxazid medication. In all patients except three, whose psychosis was activated during placebo 2, we observed a more or less pronounced improvement of the clinical symptoms, including reduction of anxiety which was retained during placebo 2. In those patients who retained clinical improvement, the urinary excretion of catechols remained low. An increase in the total catecholamine excretion occurred in those patients whose psychosis became activated and at the same time showed symptoms of increased anxiety.

TABLE 4. *Total Catechols as Related to Degree of Psychotic Activity and Grade of Anxiety*

Patient	Placebo 1			Placebo 2		
	Grade of Psychotic Activity	Grade of Anxiety	μg Total Catechols/day Avg.	Grade of Psychotic Activity	Grade of Anxiety	μg Total Catechols/day Avg.
BM	A	3	74	A	2	45
AS	A	1	34	A	1	24
FA	B	2	56	D	3	70
GF	C	2	15	D	3	70

Table 4 represents the average urinary excretion of total catechols during placebo 1 and placebo 2 in four representative patients. The biochemical results are compared with the degree of psychotic activity and grade of anxiety, the latter being scored from 1 to 3, with particular respect to the categories vasomotor hyperactivity, functional motor disturbances, reflex activity, and hypersensitivity; 1 standing for no apparent or only little anxiety, and 3 for marked anxiety; while 2 represents the intermediate state. During placebo 1, anxiety was most pronounced in *patient BM*. His psychosis was not apparently active, and total catecholamines were highest in that patient. Psychotic activity was greater in *patients FA* and *GF*; but the degree of anxiety was lower and the catecholamine level lower. During placebo 2, anxiety was less marked in *patient BM* than during placebo 1. This is reflected in a lower excretion level of catechols. In the two patients (*FA* and *GF*) who suffered an activation of

their psychosis with an accompanying increase in anxiety, the total catechols rose to the higher values seen in *BM* during placebo 1.

DISCUSSION

In this study, 835 24-hr urine specimens from 20 male schizophrenic patients were analyzed for tryptamine and total 3-indoleacetic acid. In addition 5-hydroxyindoleacetic acid was determined in 685 24-hr urine specimens from 17 schizophrenic patients. A summary of these data shows that the average daily excretion of tryptamine and 5-hydroxyindoleacetic acid was in the range of normal. The average urinary output of total 3-indoleacetic acid was slightly elevated, if compared with the results reported for normal subjects (29). All three indole derivatives display a wide range of variations, the lowest values being within normal limits, the highest, however, exceeding by far the upper normal borderline. When the biochemical results are compared with the clinical findings, it becomes apparent that all indole derivatives attain increasingly high abnormal excretion levels with augmentation of the psychosis. This rise was least pronounced for 5-hydroxyindoleacetic acid.

There are, however, individual differences in the excretion patterns of the indole compounds. During increased psychotic activity in some patients, the rise in 5-hydroxyindoleacetic acid was more marked than that of tryptamine and total 3-indoleacetic acid. This suggests that different pathways of indole metabolism may be affected to a differing degree in individual patients. The described alterations of indole metabolism during states of increased psychotic activity were observed, whether the psychosis was associated with either elevated or reduced motor activity. For this reason, the observed increases in the excretion of these indole derivatives could not be related to the state of motor activity. No correlation could be established between anxiety and urinary excretion of indoles. Those patients who displayed symptoms of marked anxiety, without apparent signs of psychotic activity, disclosed a much lower output of tryptamine and its metabolite than those whose anxiety was less marked but whose psychosis was more active.

In some patients, who suffered a sudden flare-up of the psychosis, we observed that the increase in urinary indoles occurred before the clinical symptoms became overt (6). This suggests that the described alterations in indole metabolism are not a secondary function of the psychosis. If activation of the psychosis was accompanied by marked anxiety, there was also an increase in urinary total catechols. This rise, however, in contrast to indoles, occurred simultaneously with the appearance of the clinical symptoms. Our results thus indicate that there is a correlation between the excretion of tryptamine, total 3-indoleacetic acid, and 5-hydroxyindoleacetic acid with psychotic activity, while urinary levels of catechols rather reflect different grades of anxiety.

Reserpine evoked in some of our patients a more or less short-living activation of the psychosis. In some patients it achieved, besides its tranquilizing effect, a reduction of the psychotic activity, in still other cases little effect on behavior was observed. A reserpine-induced flare-up of the psychosis was generally accompanied by a higher excretion level of the three tested indole compounds than was found before, when the mental state of the patients was in better condition. A reduction of psychotic activity under reserpine treatment on the other hand was accompanied by a decrease in urinary indoles. We observed that biochemical findings were similar in pattern, whether activation or reduction of psychotic activity occurred spontaneously or under reserpine medication. In this study, the incidence of the different degrees of psychotic activity during placebo 1 was about the same magnitude as that found during reserpine-administration.

At the same time no significant differences were found when the summaries of the biochemical data of the 2 reserpine periods were compared to placebo 1. During placebo 2, the incidence of increased psychotic activity was somewhat higher than during placebo 1. This was reflected by a higher tryptamine excretion during the second placebo period ($P < 0.05$; $P > 0.01$). Urinary total 3-indoleacetic acid and 5-hydroxyindoleacetic acid which, on the whole, were found to be less sensitive indicators of the different degrees of psychotic activity than tryptamine, were also higher during placebo 2 than during placebo 1. Their increase, however, was not significant ($P > 0.05$).

The observation that in most of our patients indole derivatives were excreted in larger amounts during periods of worsened behavior than when the psychosis was less active suggests an increased turnover of indole compounds during periods of increased psychotic activity. This relationship points to the possibility that the increased metabolism of all, or some, indoles may present an opportunity for psychotomimetic indole derivatives to be found.

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Possible relation of central amines to behavior in schizophrenic patients

SEYMOUR S. KETY[1]

Department of Psychiatry, Johns Hopkins University School of Medicine, Baltimore, Maryland

THE PRESENCE IN THE BRAIN of a number of amines, pharmacologically active elsewhere, in a differential distribution which suggests a relationship to specialized function, has evoked considerable interest and research. A number of correlations between the concentrations of some of these and behavior, and the ability of many psychotropic drugs to affect these concentrations, appear to be the results of more than chance and have formed the basis of a number of attractive hypotheses which relate one or another of these amines to normal and abnormal behavior.

The techniques available for acquiring useful information and testing hypotheses in this field have sufficient difficulties and limitations that crucial evidence for any of the hypotheses is not yet at hand (3). The concentrations of various amines which have been measured in the brain as a whole, or even in its parts, may not necessarily be indicative of the effective concentration of the material in an active state at its site of action, while the substances which have been studied may not be as potent in behavior as congeners, precursors, or derivatives as yet undiscovered or inadequately investigated. On the other hand, the behavioral observations which have been made in animals have not often been comparable to the biochemical ones in specificity, precision, or relevance to the clinical problems which have prompted them or which serve as the basis of their interpretation.

Hypotheses relating schizophrenia to alterations in central amine concentration require testing and confirmation in the naturally occurring forms of that disorder, and, in the absence of suitable animal analogues, in human beings suffering from schizophrenia. Here, direct analysis of the brain, inconclusive as it may be, is hardly possible; while changes in blood or urine need not at all reflect the chemical processes occurring within the brain. Instead, the administration of precursors of active amines, whose occurrence in the brain is known or suspected may, especially if coupled with the administration of a monoamine oxidase inhibitor, so alter the effective concentrations of particular amines at their sites of action as to produce detectable changes in mental or behavioral state, or an amelioration or aggravation of specific symptoms of schizophrenia, if there is any relationship between them. In studies in lower animals such procedures have provided useful insights (1, 2, 10) but their usefulness is considerably extended when applied to normal human subjects or schizophrenic patients.

The much wider range of psychometric and performance evaluation possible in man, his ability to report subjective changes, or the ability of skilled observers, well acquainted with the particular symptoms and the extent of their spontaneous variation, to detect significant change in schizophrenic patients, make such an approach especially appropriate for eliciting possible relationships between a wide variety of metabolic intermediates and normal or schizophrenic mental processes. It should be noted, however, that such procedures are more useful for developing hypotheses rather than for their validation. One cannot be certain that increased availability of a precursor necessarily results in an increase of effective concentration of its metabolic derivatives, so that failure to obtain mental or behavioral change with a particular amino acid cannot be used as evidence against the behavioral relevance of its products. On the other hand, the appearance of mental changes following the administration of a particular precursor is not attributable to a specific metabolic effect until a large number of alternative possibilities have been excluded.

The studies to be discussed have been carried out by Pollin, Cardon and myself, among others, in the Laboratory of Clinical Science at the National Institute of Mental Health. A preliminary report of the clinical results has appeared previously (7), and I have been asked to describe and comment upon them.

Iproniazid was selected as the potentiating monoamine oxidase inhibitor and was given in chronic dosage of 50 mg daily, and later 150 mg daily per 70 kg of body weight, to nine chronic schizophrenic patients. Three additional patients received a placebo instead of iproniazid. After at least 2 weeks of iproniazid administration at each level, a series of amino acids were administered orally, in substantial dosage and for 1 week each. Observations of effects on behavior and on the idiosyncratic symptoms of schizophrenia in each case were made by

[1] Present address: Laboratory of Clinical Science, National Institute of Mental Health, Bethesda, Md.

psychiatrists and nursing staff in a single- and double-blind design. Five amino acids (glycine, *l*-histidine, *l*-glutamine, *l*-phenylalanine, and *l*-tyrosine) produced no significant mental or behavioral change when administered in dosage of at least 20 g daily for a week, even when given in conjunction with a daily maintenance dosage of 50 mg of iproniazid. In two groups of four patients each, *d,l*,5-hydroxytryptophan or *l*-dihydroxyphenylalanine (DOPA) were given intravenously in daily dosage, beginning with 6 mg and increasing stepwise to as much as 108 mg in a single, rapid administration.

Three patients in each group were maintained on 150 mg of iproniazid daily. No immediate or delayed mental or behavioral change was noted up to or including the time when it was necessary to discontinue the intravenous administration because of the development of certain untoward somatic symptoms: gastrointestinal distress in the case of the 5-hydroxytryptophan, and transitory hypertension and cardiac arrhythmias in the case of DOPA. Pare and Sandler (6) reported similar lack of mental effects with somewhat lower dosages of these substances in patients with depression.

Two of the amino acids were associated in some patients with significant and reliable mental and behavioral changes. At 15 g orally per day in divided doses for a week, *l*-tryptophan produced effects in most patients on iproniazid not unlike those reported previously by Lauer and associates (4) in chronic schizophrenics, and by Oates and Sjoerdsma (5) in nonpsychotic patients. These effects are best described as sedation, followed by a feeling of drunkenness; euphoria, loss of inhibition, and freeing of associations. There appeared to be a transitory depression in some of the patients when the tryptophan was withdrawn. These effects appeared to be dependent upon the level of iproniazid employed; they were barely perceptible at 50 mg daily, and became more clear cut when the daily dose was 150 mg.

The only other amino acid which produced a consistent mental or behavioral effect was *l*-methionine at 20 g daily in divided doses. In 4 of 12 patients, this dosage was associated with an exacerbation of specific symptoms and a general activation, increasing anxiety approaching panic, an increasing flood of associations, and in one chronically mute patient, extreme garrulousness. These effects were not clearly related to iproniazid dosage; in fact, they seemed more severe on the initial trial, when the maintenance dosage of that drug was 50 mg daily, than on subsequent trials at 150 mg. These changes, although they varied from patient to patient, appeared to be fairly consistent in each and in most patients the characteristic response was reproducible although with somewhat diminished intensity on one or two subsequent administrations of methionine.

The results with *l*-tryptophan, especially their dependence upon the iproniazid level, suggest that an amine derivative of that amino acid may have psychotropic properties. Since the effects observed in the schizophrenic patients, however, were quite comparable to those which had been observed in normal volunteers, no special relevance to the etiology or pathogenesis of schizophrenia appears to be suggested.

In the latter connection, the effects of *l*-methionine appear to be more interesting. The property of methionine which prompted its use in these studies was, of course, its role in biological transmethylation, and the hypothesis that increased availability of methionine in the body could force the production of some methylated compounds which might alter behavioral state. In this connection, it is interesting to note that Axelrod has recently demonstrated the ability of certain tissues in vitro to produce, in the presence of methionine, methylated derivatives of normal metabolites, such as dimethyltryptamine, and dimethyl serotonin, both of which are hallucinogenic in man (9). Since the behavioral effects of methionine in these patients may be interpreted as representing an accentuation of the schizophrenic symptomatology, it is not difficult to formulate hypotheses relating biological transmethylation to the pathogenesis of schizophrenia. These findings are compatible with such hypotheses, although they are very far from substantiating them.

A number of alternative explanations for the observed effects of methionine must be considered. In the initial trial, methionine, which was given suspended in chocolate milk, as were the other amino acids, was associated with considerable gastric distress, nausea, and, in some instances, vomiting. It is also capable of causing a mild acidosis. The possibility that the mental and behavioral effects were in response to either of these side effects appears to have been ruled out in the same patients by administering this amino acid in the form of capsules. The gastric distress was then negligible, but the mental and behavioral changes were, nevertheless, apparent. Furthermore, ammonium chloride given in enteric coated tablets produced comparable degrees of acidosis and gastric distress, with none of the behavioral effects noted with methionine.

There is the possibility that the effects of methionine are nonspecific "toxic" effects and unrelated to schizophrenia. We have begun to test this hypothesis by administering comparable doses of methionine to normal volunteers. In five such trials, without the possible potentiating effects of iproniazid, a few of the subjects reported barely perceptible subjective feelings of light headedness, which compared qualitatively and quantitatively with the effects produced by small amounts of alcohol. What remains to be determined, however, is the effect of methionine in a larger series of normal volunteers pretreated with iproniazid.

It is possible that the marked effect of the methionine-iproniazid combination in one third of the schizophrenics represents a peculiarly schizophrenic response to a subjective state, which, in itself, is not unlike that produced in normals. This appears to be unlikely, in view of the results in these patients with the tryptophan-iproniazid combination. Such combination produces in normals a sense of light headedness and intoxication resembling that of alcohol, and the effects produced in

schizophrenics are not different in nature or degree. Summerskill and his associates (8) have produced mental and behavioral changes similar to those which we have seen in schizophrenic patients by giving large doses of methionine to patients with severe hepatic insufficiency. In their study, however, stupor and serious neurological derangement accompanied the methionine administration. Although the presence of reduced hepatic function is not uncommon in unselected populations of chronic schizophrenics, it appears unlikely that these carefully selected patients, with no evidence of hepatic insufficiency by standard laboratory tests, would be comparable to Summerskill's patients; nevertheless, that possibility remains to be further evaluated.

Our efforts are now directed at studies to determine whether a significantly higher percentage of schizophrenic patients show qualitatively and quantitatively different behavioral responses to methionine and iproniazid from those produced in nonschizophrenic individuals, and, if so, the mechanism by which the effects are produced, and their possible relevance to schizophrenia.

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Discussion

NICHOLAS J. GIARMAN

Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut

When DR. HIMWICH AND LATER DR. DEWS, called me about appearing as a discussant on this program, I pleaded ignorance about the effects of hallucinogenic agents in man. They persuaded me, however, that what was needed was an appraisal of the data in terms of sound pharmacological principles (and they included in this, I am sure, physiological and biochemical principles). Their assurances were responsible for my not realizing then, as I did a few moments later, that there are very few sound pharmacological principles which one can apply to any classification of drugs affecting the CNS. However, this has not stifled discussion in other such symposia and will not dissuade me.

I doubt that anyone present today would argue strongly against the proposition that the mechanism of action of psychotropic drugs in general is still largely unknown. It is true that a large number of behavioral and chemical correlates of drug-induced changes in the brain have been accumulated, and some of these have been embodied in certain seductively attractive hypotheses. Regardless of what formulation one takes of the problem, the evidence is strong, even if mostly presumptive, that there is a correlation between biogenic amines in the brain and drug-induced changes in behavior. I would point out that the correlation is usually made with respect to the size of the cerebral store of one or another endogenous amine; and this is attractive, because it is believed to be associated in some way with neurohumoral regulation of interneuronal communication.

In this view, synaptic transmission is considered a key factor in the neurochemistry of cerebral function. This, I think, is a useful premise. However, the general idea that there is a causal relationship between the store of amines and behavioral change, no matter how attractive, has little direct evidence supporting it and some evidence against it. One of the major problems, it seems, is an operational one of finding the most direct methods of obtaining information to achieve rigorous support of such an hypothesis.

Unfortunately, the data so far accumulated have come from fairly indirect studies; and this represents one of the drawbacks of some of the work presented today. I say this in all humility, as one who can pull a rat from its cage without worrying about precise diagnostic analysis, how to get a sample of brain, the problem of suggestion, and the like. In short, I realize fully the difficulties of clinical experimentation. But this does not get us away from the fact that metabolites of various amines in the urine, for example, represent at best a very minor contribution from cerebral metabolism, and a very large contribution from extraneural metabolism.

The experimental approach is always ingeniously being improved, and we have today a much more sophisticated understanding of the problem than we had a few years ago, thanks to contributions such as those we have heard today.

The problem of 6-hydroxylation of indolealkylamines is indeed a fascinating one, and Dr. Szara and co-workers have made a good start toward establishing a 6-hydroxyindole amine of endogenous origin as a psychotoxin of importance. These investigators have already established the psychotomimetic potency of such derivatives (as 6-hydroxydimethyltryptamine and 6-hydroxydiethyltryptamine); and it is, therefore, possible to conceive of a high circulating level of a similar compound and its presence in the brain as representing a psychotoxic basis for mental disorder. Perhaps related to this is a recent finding of Dr. Sprince of high levels of 6-hydroxy skatole in the urine of schizophrenics (9).

Dr. Szara's presentation raised some questions in my mind: *1*) 6-Hydroxyindoleacetic acid represents an important metabolite in such a study. It was not clear to me whether it was estimated and included in the data on 6-hydroxy indole metabolites. If it was not, it would seem to be a serious omission in any attempt to correlate rate of metabolism with variation in response.

2) Would not an inhibitor of monoamine oxidase (MAO) be expected to potentiate the psychotomimetic potency of DMT and DET by sparing the 6-hydroxy amine from oxidative deamination and allowing it to accumulate? The same would be true for inhibition of glucuronide formation. Here, however, I may be falling into an error in thinking, common in this field, which should be pointed out and corrected by the pharmacologist. The danger lies in arguing about central mechanisms from a constellation of actions of a substance on peripheral organs. For example, the absence of a pro-

nounced behavioral effect following BOL does not rule out serotonin as an agent, possibly implicated in the central actions of other 5-HT anatagonists, like LSD.

3) Along this same line, is not α-methyltryptamine an MAO inhibitor itself? What happens to the brain amines after this substance is administered and could some of its effects be due to such changes?

4) Finally, couldn't we circumvent the necessity of speculating on how 6-hydroxy tryptamine might get into the brain, by assuming, or possibly showing, that it is made in the brain from the tryptamine already there?

Dr. Kety has, as usual, obtained some fascinating findings from his well-planned and carefully executed studies. One might speculate, along with Dr. Szara, that the methionine-induced effect is due to methylation of Szara's 6-OH psychotoxin, allowing it, thereby, to enter the CNS. It might be of interest to test this possibility by determining whether methionine, as an adjuvant, uncovers any psychotomimetic action which 6-hydroxytryptamine might have. Another possibility which arises in my mind is that the effects of methionine may have been mediated by some abnormal metabolite of methionine, similar, for example, to methionine sulfoximine, which is well known to cause marked alteration in brain excitability. I would like to hear Dr. Kety's views on these points.

The results and interpretations of Doctors Brune and Psheidt interested me greatly, because they touch upon some of our recent work, which I will speak of in a moment. While this and other investigations of theirs suffer from the difficulty of extrapolating from urinary products to brain metabolism, they have hit upon an interesting general point, which several observers in this field have been speculating about, namely, that the size of the total cerebral store of an amine may not be nearly as important as its sub-cellular status and form in the brain, for interpreting drug-induced behavioral change.

Figure 1 shows schematically our current concepts of these various sub-cellular forms. We visualize two bound forms of the neurohumor: *1*) the inactive storage form which is granule-bound and inaccessible to the inactivator enzyme; and *2*) the active form, represented by the complex of the neurohumor with its receptor protein.

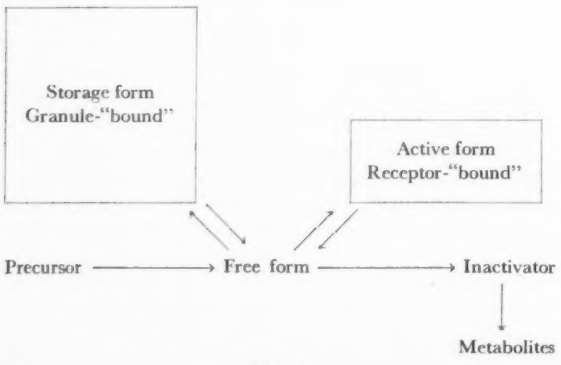

FIG. 1

TABLE 1

Treatment	No. of Animals	Mean 5-HT (mμg/Whole Brain)			% "Free"	P/S Partic/ Supernate
		Partic. "Bound"	Supernate "Free"	Total		
None	62	389±89*	158±36*	547	29	2.5
Chlorpromazine, 25 mg/kg—75 min	12	300†	247†	547	45	1.2
Reserpine, 5 mg/kg—4 hr	14	88†	61†	149	41	1.4
Isoreserpine, 5 mg/kg—4 hr	4	275†	129	404	32	2.1
α-Methyl-DOPA, 100 mg/kg—2.5 hr	12	267†	182	449	41	1.5
Phenobarbital, 100 mg/kg—2.5 hr	5	450†	325†	775	42	1.4
Methylparafynol, 300 mg/kg—15 min	7	321	139	460	30	2.3

* Standard Deviation. † These values differ significantly from the control value at or beyond the 5% level of confidence.

Dr. Schanberg and I have shown that the bulk (70%) of endogenous 5-HT in rat brain exists in these forms, mainly, it may be assumed, in the storage form (3). There is also the "free" form, usually equated with the active material, but in reality representing a transition state; potentially active, potentially storable, and potentially destructible.

A drug, conceivably, could influence the amount of the free form and thereby alter responses evoked by the active complex. In this sense, drugs might: *a*) inhibit the inactivator; *b*) inhibit or activate the synthesizing enzyme; *c*) block reception at the receptor; *d*) cause release from the storage site, and ultimately deplete the store; and *e*) promote binding at the storage and receptor sites, or both.

Moreover, these effects of drugs are not mutually exclusive; and because similar molecular forces may operate at several of these sites, it is conceivable that a drug may act at more than one site.

We, consequently, felt that a ratio of the two bound forms to the free might be a more reliable correlate of drug-induced behavioral change than size of the total cerebral content of neurohumor has proved to be. By high-speed centrifugation of rat brain homogenates in isotonic sucrose (0.25 M) it is possible to get reliable and reproducible ratios of "bound" to "free" 5-HT, which have responded in a predictable manner to certain classifications of CNS drugs. The 5-HT in these experiments was extracted into acetone and taken to dryness. These extracts were kept at −16 C until assayed on the heart of the clam, *Mercenaria* (*Venus*) *mercenaria*.

Table 1 shows results obtained with drugs which represent various sub-classes under the broad heading of CNS depressants. We see first the two separable and distinct forms of 5-HT in the brain (with 29% free).

Many control studies have shown us that this is a labile and artifactitious system and is probably not indicative of in situ conditions. Nevertheless, as you will see, it is productive of interesting and predictable correlations. Furthermore, it provides data differentiating drugged and nondrugged states and, what is more important, differentiating between classifications of CNS drugs. The following points demonstrate this differentiation:

1) Reserpine, e.g., does what many have suspected it does in the brain; namely, it causes a greater depletion of stored serotonin (5-HT) than of the free form. This results in a proportionally greater amount in the free form and a "bound"/"free" (P/S) ratio of 1.4, instead of 2.5.

2) It may be seen, further, that some of the other potent depressants, regardless of their effect on total cerebral level of 5-HT, cause a similar shift of P/S ratio toward 1.0, including: α-methyl DOPA, which inhibits synthesis and decreases the total slightly; phenobarbital, which raises the total by 42%; and chlorpromazine, which does not alter the total.

3) It is interesting that isoreserpine, which is practically devoid of CNS activity, causes little change in either total 5-HT or in the P/S ratio.

4) Finally, another depressant, methylparafynol ('Dormision') which we have shown (6) to be associated with considerable elevations (70–90%) in brain acetylcholine (ACh) when used in anesthetic doses, has no effect on intracellular distribution of 5-HT. This is not surprising and indicates that the ACh system should not be neglected in our thinking and investigations. As a matter of fact, the powerful psychotomimetic drug, 'Sernyl,' given in high doses (20 mg/kg) to rats, also causes an elevation in brain ACh, and no change in the 5-HT system (Pepeu and Giarman, unpublished data).

Let us now turn attention to some drugs which produce essentially the opposite behavioral effects. These are the antidepressants, CNS excitants, euphoriants, and psychotomimetic agents. Some results are summarized in Table 2.

TABLE 2

Treatment	No. of Animals	Mean 5-HT (mμg/Whole Brain)			% "Free"	P/S Partic/ Supernate
		Partic. "Bound"	Supernate "Free"	Total		
Iproniazid, 100 mg/kg—12 hr	17	996*	485*	1481	33	2.1
β-Phenylisopropyl-Hydrazine, 10 mg/kg—18 hr	16	1050*	343*	1393	25	3.1
Imipramine, 15 mg/kg—75 min	4	506*	200	706	29	2.5
LSD-25,† 520 μg/kg—20 min	8	531*	156	687	23	3.4
None	62	389	158	547	29	2.5

* These values differ significantly from control values at or beyond the 5% level of confidence. † These values, estimated by bio-assay, have been confirmed by similar experiments in which the 5-HT was determined spectrophotofluorometrically (unpublished data of D. X. Freedman).

Here we see at first glance that all these agents cause a rise in total 5-HT, but, simultaneously, either have no effect on or elevate the P/S ratio above 2.5. In collaboration with Dr. Freedman, we have found that the most striking agent in this group is LSD, which apparently causes an enhanced binding of 5-HT, since all of the rise in total 5-HT (in this case, 25%) can be accounted for in the "bound" fraction (2). Dr. Freedman has shown that this effect is more marked in reserpinized animals and can be seen with threshold doses for autonomic and behavioral effects of LSD (1). Whether this is a characteristic of other psychotomimetic substances needs to be investigated.

Another interesting aspect of these data is that the large increase in total 5-HT following inhibition of MAO (by either iproniazid or β-PIH) was distributed between the particulate and non-particulate compartments so as to maintain a relatively normal P/S ratio. This might suggest a dynamic steady-state relationship between 5-HT in the stored reserves and that free in the cytoplasm.

This technique has uncovered one final interesting interrelationship: that of reserpine with MAO inhibitors. Table 3 shows some of these data. A number of reports, originating with Pletscher (8), have indicated that iproniazid interferes with reserpine-induced release of catecholamines and 5-HT. Pletscher and Paasonen (5), e.g., have observed an interference by iproniazid with reserpine-induced release of 5-HT from platelets. Green and Sawyer (4) have seen an interference by tranylcypromine with reserpine-induced release of catecholamines from brain granules; and in our laboratory, with Doctors Pepeu, Roberts and Schanberg, we have ob-

TABLE 3

Treatment	No. of Animals	Mean 5-HT (mμg/Whole Brain)			% "Free"	P/S Partic/ Supernate
		Partic. "Bound"	Supernate "Free"	Total		
None	62	389	158	547	29	2.5
Reserpine, 5 mg/kg—4 hr	14	88	61	149	41	1.4
Iproniazid, 100 mg/kg—12 hr	17	996	485	1481	33	2.1
Iproniazid (6), followed by Reserpine (3)	16	1001	541	1542	35	1.9
PIH*, 10 mg/kg—18 hr	16	1050	343	1393	25	3.1
PIH (2), followed by Reserpine (3)	20	665	339	1004	34	2.0
Imipramine, 15 mg/kg—75 min	4	506	200	706	29	2.5
Imipramine (8), followed by Reserpine (3)	12	73	29	102	28	2.5

* I-Phenylisopropylhydrazine.

served a strong interference by iproniazid with release of catecholamines from guinea pig's atria (7). The data in Table 3 strikingly show the anti-reserpine action of iproniazid. Particulate-bound 5-HT, which, ordinarily is 77% (389 mμg to 88) depleted by reserpine, remains unchanged when the animals have been pretreated with iproniazid. β-Phenylisopropylhydrazine (PIH) was less potent in this anti-reserpine effect, allowing reserpine-induced depletion to occur up to 37% (1050–665 mμg). Since PIH was used here in a dose roughly equivalent in MAO-inhibiting potency to that of iproniazid, the anti-reserpine action cannot be due solely to inhibition of MAO.

If there is a moral to this story, it is that the direct approach of studying bound and free neurohumors can, *a*) substantiate some of the hypotheses already proposed for the action of certain psychotropic drugs, e.g., the action of reserpine; and *b*) yield data useful in the development of new and testable hypotheses based on; e.g., enhancement of binding of 5-HT by LSP and, inhibition of amine-release by iproniazid, and possibly other MAO inhibitors.

While these data are to be considered purely as correlations, such correlations may be indicative of underlying mechanisms. For instance, LSD-induced behavioral changes are, no doubt, related to a number of biochemical mechanisms. Our proposition is that it may be fruitful at this stage to consider interactions with particulate-bound 5-HT as a reflection of interactions at a complex of receptor sites.

We must, however, guard against what A. N. Whitehead called the "fallacy of misplaced concreteness." When we reason deductively about complex affairs, we nearly always leave out many things, both things forgotten and things unknown. This leads to erroneous conclusions. Only experience can teach us the immenes complexity of interactions among many factors. This is sufficient reason for us to bring this Symposium to an end; return to the laboratory, and do more work.

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Introduction

BENNETT J. COHEN
University of California School of Medicine, Los Angeles, California

THE ANIMAL CARE PANEL and the Federation welcome you to this Symposium, the first of its kind held at a Federation meeting. The session has been organized to present a sampling of current work in laboratory animal medicine, and to illustrate to biologists some of the developments and problems in this challenging new field.

It is now generally accepted that the care of laboratory animals is a fundamental consideration in planning and conducting research. At this session some of the implications of the word "care" will be considered. The problems of selection and quality of research animals, of technical and academic training of animal care specialists, and of housing the laboratory species are areas of basic concern not only in laboratory animal medicine, but to all biologists. They bear directly on the success of any research program involving animals. To some extent, every one who uses animals in research should be expert in laboratory animal care. It is appropriate to devote a session to this subject at this Federation meeting, the largest gathering of biologists in the world.

There is another important reason for this session. As all biologists know, the problems of care and use of animals are much in the public eye these days. A political issue has been created around this subject, and as a result the American scientific community is faced with a major crisis.

Legislation is again pending in Congress to license scientists and laboratories engaged in Federally supported research. Under the guise of providing for the humane care and treatment of laboratory animals, bills sponsored in the House by Representative Martha Griffiths and Representative Morgan Moulder; and scheduled for reintroduction in the Senate by Senator John Sherman Cooper would establish complex Federal regulatory machinery to direct and control animal experimentation.

The scientific community is united in opposition to these destructive legislative proposals, which would only restrict and hamper needed research without contributing to the advancement of laboratory animal care. We have every reason to press for their defeat. However, we must do more than simply oppose these bills. We have an obligation, as biologists, to inform Congress, the public, and ourselves regarding the needs and the problems of modern animal experimentation. In addition, we must work constructively to assure the highest scientific and ethical animal care standards. To these objectives, this Symposium is dedicated.

We should encourage Congress and the public to assist us in giving rational meaning to the word "humane" as applied to the care and use of the laboratory species of animals. Several constructive approaches are available which avoid the bureaucratic restrictions of pending legislation. For example, greater support of research and training in laboratory animal care is needed. Programs for the improvement of existing animal facilities and construction of new facilities would be welcomed; the factual information in this important area of biology should be more widely available.

This Symposium touches on all of these approaches. The participants hope that you will find their presentations of interest and of value. They welcome your comments and questions during the discussion period to follow.

Choice of experimental animals for research

BODIL SCHMIDT-NIELSEN[1]
Department of Zoology, Duke University, Durham, North Carolina

A FEW YEARS AGO, Dr. P. F. Scholander, addressing a group of comparative physiologists said: "To the medical physiologist, man is made of dog heart and kidneys, frog muscles, rat intestines, fruit fly genes, etc." He added: "It has always been a wonder to me why the ordinary laboratory animals are considered so particularly anthropoid." Scholander, with his usual sense of humor, had pointed to a rather sore spot in physiological research; namely that the variety of animals generally used is quite limited, and that deductions as to physiological mechanisms are sometimes made from one species to another without sufficient justification. In the present discussion, I would like to plead for a wider and more discriminating choice of experimental animals.

EXPERIMENTAL ANIMALS PRESENTLY CHOSEN BY PHYSIOLOGISTS AND TYPE OF INVESTIGATIONS FOR WHICH THEY ARE USED

To get an idea about the kind of animals currently used by physiologists, I surveyed the abstracts in Federation Proceedings last year. There were nearly 700 studies by physiologists. In over 70% of these, dogs, rats, man and monkeys were used, with 28, 22 and 21%, respectively (Fig. 1). Other animals occurred surprisingly seldom. Rabbits, mice and frogs were each used in less than 5% of the studies. The remaining 8%, which are not shown on the graph, included 34 different species of animals less commonly employed in the laboratory (Table 1).

In Fig. 2 is shown the type of experiments conducted on dogs, rats, primates, and cats. Over 50% of the dog experiments were concerned with circulation. The rat was most frequently used for endocrinological and nutritional research. In general, it seems to be a very versatile laboratory animal, used in a variety of different studies. Man and monkeys were used most predominantly in respiration studies, but, also, in many other experiments. The cat was used almost exclusively in neurophysiological experiments.

If we investigate the reasons for the particular choice of animal made by the investigators, we find that they are mostly practical and based on very valid considerations. Since it is imperative in physiological research that the animal be healthy and in the best possible nutritional condition, it is natural to choose animals *1*) that can be maintained in captivity; *2*) for which adequate facilities are present in practically all laboratories; *3*) for which we are thoroughly familiar with the nutritional requirements; *4*) for which we have some knowledge of the common diseases and the cure for them. Furthermore, it is important that *5*) the anatomy is well worked out. The size of the animal is often an important consideration. In studies concerned with circulation and kidney function, the rat is often too small to work with conveniently, so the dog is chosen instead.

Another important consideration is that the animals can be trained to the point where ordinary quiet handling does not change physiological reactions. In this respect, the dog is particularly good, because it can with appropriate rewards be taught to cooperate with the investigator. On the other hand, it is a curious fact that a highly domesticated animal, like the rabbit, is exceedingly sensitive to any kind of handling. Grant (7) noted that if a rabbit is placed in a laboratory in an open wire cage, which is small but completely comfortable, the body temperature of the rabbit decreases, and can fall 2–3 degrees in 30–90 min. This is in spite of the fact that the rabbit sits perfectly quiet and shows no outward sign of distress. Forster (5) found that if cigarette smoke is blown over the nose of the rabbit, it stops breathing and a pronounced bradycardia, accompanied by oliguria, sets in. The rabbit in this respect reacts much as a diving animal, as, for example, the seal. Hansen and Brezowsky (8) found that warm humid weather increased the inulin sensitivity in rabbits, while a dry atmosphere decreased the sensitivity.

The wide use of man, which, incidentally, was a surprise to me, has in many instances been determined by the fact that the investigator can communicate directly with the experimental subject, and that the subject (sometimes) is willing to cooperate with the investigator.

In 8% of the studies, a variety of animals were used and the choice was based on a different set of considerations (Table 1). Invertebrates, including paramecia, planarians, lobsters, and insects, were used in studies, including behavior, nerve and muscle physiology, renal function, endocrinology, and sensory physiology. Nonmammalian vertebrates, fish, alligators, penguins, tadpoles, frogs, and lizards were used for metabolism, be-

[1] Established investigator of the American Heart Association.

havior, intestinal absorption, and neural physiology. Mammals, including wild and domesticated mammals, squirrels, beavers, desert rodents, pigs, and hamsters were used for hibernation studies, renal function, blood pressure, and blood brain barrier.

I have tried to classify the major reasons for these choices, and they are shown in Table 2.

ANIMALS IN WHICH THE ANATOMY IS PARTICULARLY FAVORABLE FOR STUDYING CERTAIN PHYSIOLOGICAL FUNCTIONS

August Krogh once said that if you look carefully you can always find an animal that seems to have been designed by nature for the investigations you have in mind.

Some of the most spectacular progress has come about in modern physiology following the discovery of the right kind of animal. I should like to illustrate this point with some examples.

The enormous progress that has taken place in the last two decades in neurophysiology is to a considerable degree based on the discovery (or rather rediscovery) by Young, 1934 (21, 22) that there are two very large nerve cells in the central nervous system of the squid Loligo and its relative Sepia. The first discovery was made by Williams (20) as early as 1809, but his account was not amplified or even mentioned until Young accidentally rediscovered these large nerve cells with an axon diameter of 1 mm, and at the same time realized the great advantage these giant axons offered to the study of nerve conductance and histology.

In the early 1920's, E. K. Marshall, together with Vickers, found that the renal tubules in the dog are capable of actively secreting phenol red into the tubular lumen (11). The evidence was convincing, but at that time Cushny's theory on the formation of urine was still prevailing. It was believed that only two processes, glomerular filtration and tubular reabsorption, were involved in urine formation. A. N. Richards, who himself had done brilliant work in kidney physiology, was at that time opposed to the notion that any substances are secreted into the tubules. Marshall, then in a search of

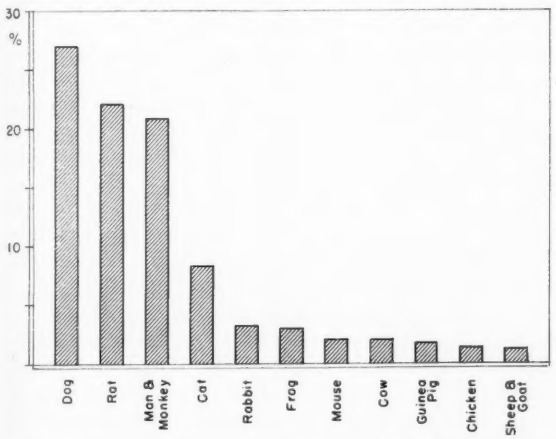

FIG. 1. Frequency of use of various animals in physiological experimentation. *Ordinate:* percentage of abstracts in Federation Proceedings, 1960, reporting use of the animals. (Schmidt-Nielsen, *Am. Heart J.* 62: 579, 1961, C. V. Mosby Co.)

TABLE 1

Invertebrates	onmammalian Vertebrates
Paramecium	Fish
Hydra	Necturus
Starfish	Tadpole, frog
Pecten	Lizard
Sea cucumber	Alligator
Squid	Turtle
Planaria	Snake
Phascolosoma	Pigeon
Caterpillar	Penguin
Southern army worm	Chicken
Diptera	
Hymenoptera	Mammals
Coleoptera	Hamster
Cockroach	Squirrel
Firefly	Beaver
Lobster	Psammomys
Crayfish	Goat
Crab	Pig

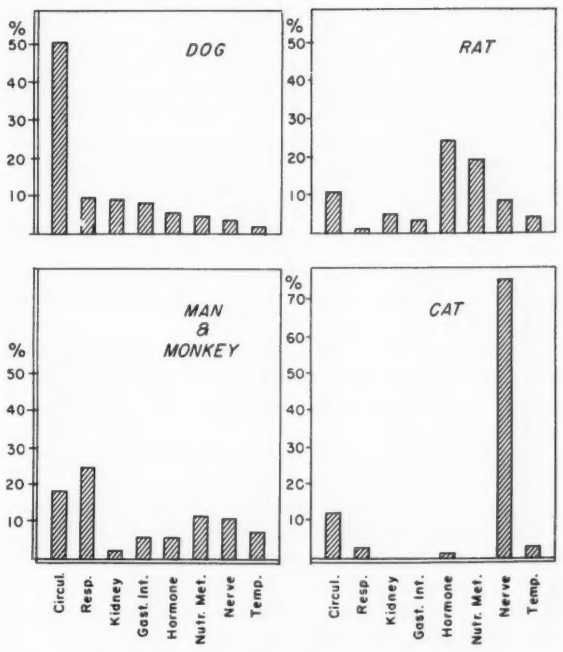

FIG. 2. Investigations in which dog, rat, primate and cat have been used. *Ordinate:* Percentage of the total number of abstracts for each animal concerned with the various types of investigations.

TABLE 2

1. Animals in which the anatomy is particularly favorable for studying a certain function
2. Animals in which a physiological function has been developed to an extreme (often through adaptation to special environments)
3. Comparative studies of animals including lower vertebrates as well as invertebrates

the literature, came across the animal ideally suited for his experiments. This was the goose fish, or angler fish, *Lophius piscatorius*. Huot in 1902 (9) and Audige in 1910 (1), described the kidneys of these fish as having neither an arterial supply nor glomeruli. Marshall and Grafflin (12) writes: "When one considers the great amount of work which has been done on the frog's kidney, and the importance attached to these studies on account of the double blood supply to the tubules, it is quite surprising that little work has been done either to confirm and extend these extremely interesting anatomical observations of Huot and Audige, or to study the function of such a pure tubular kidney."

A functional study of the kidney of *Lophius piscatorius* was commenced in the summer of 1926, and observations were also made in an attempt to confirm the essential findings of Audige.

In the summer of 1928, Marshall was in Woods Hole, where he presented his work showing beyond doubt that the renal tubules in the aglomerular kidney of the goose fish are capable of secreting not only phenol red but a number of other substances. In the discussion that followed, Richards exclaimed: "Thank God! Marshall has at last found one beast in the whole animal kingdom that fits in with his theory."

The studies by Marshall on the goose fish kidney were followed by a long series of studies by other investigators, particularly by Forster and his collaborators (5). The understanding of renal function has been considerably advanced through the studies of the aglomerular kidney in the goose fish.

It should be mentioned here that another anatomical pecularity in the goose fish makes it particularly suited for the study of neurosecretion. Carlisle in 1957 (3) pointed out: "The pituitary stalk of the angler, or goose fish, *Lophius piscatorius*, is notable for its length, which may exceed 3 cm in a large fish. It is only about 50 μ thick and is accompanied by a hypophyseal portal vein. It is, thus, eminently suited for visual and electrical observations on the functioning of the pituitary stalk.

"With the normal light microscope, intra-axonal objects could be observed migrating down a proportion of the axons, in most preparations. Some of these could be interpreted as mitochondria, but others, rather larger and of mulberry appearance, appeared to be conglomerations of neurosecretory material. They were quite unlike anything which I have observed in ordinary nerve fibres. These objects moved towards the pituitary at a rate of about 100–200 μ per minute. The movement ceased two or three minutes after removing the preparation from the body."

The teleost fish, in general, also seems to be designed by nature for the study of another physiological function. This was pointed out by Buddenbrock (2) in his discussion of the Islets of Langerhans. Buddenbrock writes: "In the teleost fish, a very special arrangement is found. In many, the islets are not numerous and small, but are few and very large; often one large main islet and some smaller islets are found. They are separated from the main part of the pancreas and are easy to isolate and remove. The first to utilize these in physiological experiments was Diamare (1905). In spite of this very useful property of the islets, they have not played any important role in the discovery of insulin." It appears that if advantage had been taken of this knowledge, the discovery of insulin might have come about sooner than it did.

In renal physiology, the fact that the lower vertebrates, i.e., all vertebrates except mammals, have a renal portal circulation has been utilized by several investigators. Sperber (18) showed in 1945 that a large fraction of the blood from the leg vein of the bird goes directly to the kidney tubules by way of the renal portal vein. He found that when substances secreted by the renal tubules are injected into the leg vein on one side, these substances appear in the urine from the kidney or the same side much earlier than from the other kidney. This fact has been utilized in many studies of tubular secretion.

The fact that certain desert mammals have a very long papilla renis has been utilized in kidney studies by Ullrich, Gottschalk and myself. In the hamster, the papilla renis projects into the ureter and, thus, by exposing the kidney and opening the ureter, one can gain access to the papilla in the live anesthetized animal. Jarausch and Ullrich, using a very elegant technique, were able to place microcatheters of polyethylene tubing into the collecting ducts of the papilla, and to collect urine samples at varying distances from the tip of the papilla. They were, thus, able to determine quantitatively the reabsorption of water and solutes taking place along the collecting ducts (10). The elegant micropuncture studies on the loops of Henle from the papilla of the hamster that Gottschalk has performed were reported by him in the Bowditch Lecture for the Physiological Society last fall (6). In analysis of the distribution of solutes in kidney tissue, the long papilla also offers special advantages (14).

ANIMALS IN WHICH A FUNCTION HAS BEEN DEVELOPED TO AN EXTREME

In animals that are specialized to live in certain adverse environments, we often find that physiological functions have developed to an extreme that we do not find in related species living in more moderate environments. Such animals offer particular advantages to physiological studies, not only because the anatomy might be favorable for experimental procedures, but also because the basic physiological processes can be better evaluated when the range and the flexibility of the function are studied.

We can also study adaptation for a different reason, which to the physiologist often is the most important, and that is to find out how nature has solved a difficult problem.

The following examples will mainly show the enormous range in physiological adaptation but may also point to some interesting animals that may be suited for further experiments.

The Chironomid larvae (the larvae of a small fly) can live under the most diverse conditions, and the study of how they do so is fascinating. Scholander, Flagg, Hock and Irving, 1953 (16) studied the Chironomid larvae in Alaska. In the Winter these larvae were found in the frozen black mud in shallow pools. The temperature taken in bore holes showed this habitat to go below −20 C. Pieces chopped out of the frozen mud were placed outdoors near the laboratory for several months, and subjected to temperatures frequently as low as −40°. The larvae could be thawed out, and they would still be alive and perfectly healthy. Most animals that we know of do not tolerate any degree of ice formation in the body fluids. Thus, in the hibernating mammals, the body temperature is carefully regulated above freezing, and the cold blooded vertebrates do not survive freezing, as far as is known. And yet, in the Chironomid larvae, at these low temperatures, 90–95% of the total body water was frozen. In spite of this, respiration continued, although it was considerably reduced at the lower temperatures.

Another peculiar environment in which certain Chironomid larvae are able to thrive is saturated salt lakes. Surovow (19), 1908, reported that he found various small animals in Lake Bullock, near Fort Alexandrowsk. The lake has a total salt concentration of 28.5% and a sodium chloride concentration of 18.3%. He found not only Chironomid larvae, but also Oligochaeta, crustaceans, and Rotatoria. Osmoregulation has been studied intensively for marine and fresh water animals, but, as far as I know, very little work has been done on the osmoregulation of several of these.

Another example of an animal adapted to an adverse environment is the lung fish, "Kamongo", which was studied by Homer Smith in 1928 (17). Its habitat in the equatorial region of Africa is subject to heavy rains, which alternate with protracted periods of drought. During the arid period, the fish estivates in cocoons in the mud. When the water recedes, it squirms into the mud, following the subsoil water down to a depth of 12 to 18 inches, keeping a small blow hole open at the top of the cavity. It can remain in this cocoon for at least 3 years, and possibly much longer.

Among the several interesting observations that Smith made on the lung fish during and after estivation, was the fact that their metabolic rate drops by 50% within a week, and then decreases more slowly, and within 3 months reaches a low level of 10–15% of that in the fed and active state. Urinary secretion stops altogether, and metabolic waste products are stored in the body. It was found that the urea concentration of fish that have estivated for 3 years was 3–4%. To the medical physiologist, who knows the dangers of anuria in man, this is an amazing fact.

Smith observed: "If the lung fish is capable of learning by experience, it seems reasonable to suppose that this is all erased during its long sleep. It can be suspected (laboratory observations afford no proof) that the creature has to learn how to eat again. Time, as it were, has passed it by, and one can seriously ask if it has grown older with the passing years." Thus, the lung fish appears to be an interesting subject for further studies, both in behavior as well as in physiology.

COMPARATIVE STUDIES OF ANIMALS, INCLUDING LOWER VERTEBRATES AND INVERTEBRATES

The fundamental significance of physiological functions is often much better understood when viewed on

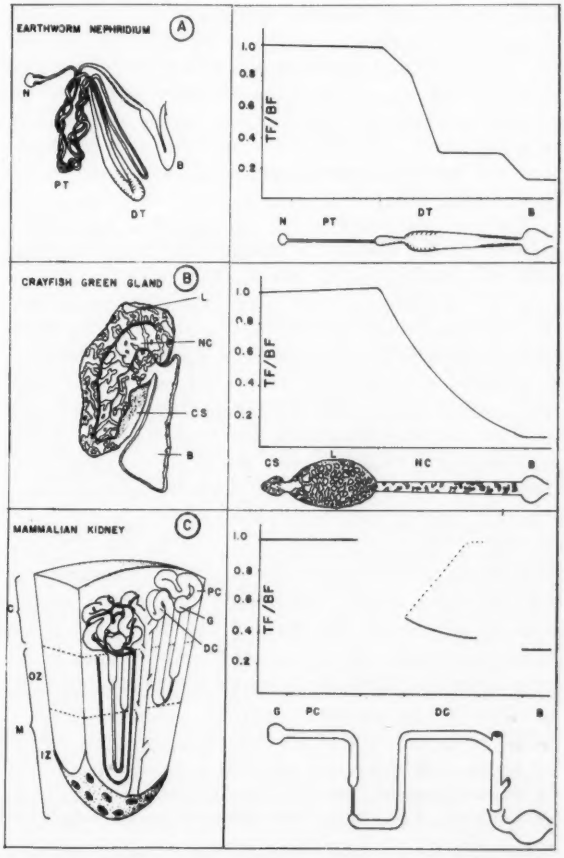

FIG. 3. Osmotic concentration along the excretory tubule of the earthworm, the crayfish and the mammal [reprinted with the permission of the American Heart Journal (15)]. The figure emphasizes the fact that two of the primary functions of the kidney are to conserve sodium and excrete water. *A: Earthworm nephridium. PT*, proximal tubule; *DT*, distal tubule; *B*, bladder. The osmotic concentration in the tubular fluid divided by the osmotic concentration of the body fluid are plotted against the site of the puncture on the nephridium. *B: Crayfish green gland. CS*, coelomic sack; *L*, labyrinth; *NC*, nephridial canal; *B*, bladder. Determinations of the chloride concentration in the various parts of the green gland are presented as the ratio between the concentration in tubular fluid and the concentration in the body fluid. *C: Mammalian kidney. G*, glomerulus; *PC*, proximal convolutions; *DC*, distal convolutions; *C*, cortex; *M*, medulla; *OZ* outer zone of the medulla; *IZ*, inner zone of the medulla. Along the *ordinate* are given the osmotic concentrations in the tubular fluid divided by the concentration in the blood. *Solid line* represents the data from rats producing a hypotonic urine. *Dotted line* represents data obtained in rats during low urine flow when the urine was hypertonic.

a broad comparative basis than if observed only in one species or even one order of animals. The well-known fact that the metabolic rate of mammals per unit of body weight decreases when body size increases was long interpreted as a reflection of the fact that heat is dissipated from the surface of the animal. Since small animals have a larger surface area in relation to their volume, their metabolic rate should naturally be greater than in larger animals. However, when Zeuthen (23) studied the relationship between metabolic rate and body size in cold blooded animals, including several groups of invertebrates, the same relationship between metabolic rate and body size was found. It was obvious that the rate of heat loss from the body surface of the animal was not the only decisive factor determining metabolic rate, and that the relationship between body size and metabolic rate is probably of a much more fundamental nature.

The fact that some of the primary functions of the kidney, besides nitrogen excretion, are water excretion and salt conservation is not obvious from the kidney function of mammals. In some desert mammals the concentration of salt in the urine can be two to three times as high as in sea water, and the amount of urine produced is very low (13). In the crayfish, on the other hand, which lives in fresh water, the urine flow is large, and the urine is practically salt free. In spite of this enormous difference between the urine normally produced by the two kidney types, the basic function of the different parts of their kidneys are essentially the same. In Fig. 3, the osmotic concentration in the different parts of the tubule is shown for the earthworm, the crayfish, and the mammal. The fluid in the proximal tubule remains isotonic to the body fluids, while the distal tubule, which by the way is structurally similar in all three types of animals, performs the same basic function: salt is reabsorbed leaving the fluid in the distal tubule hypotonic to the blood. This function is still performed by the distal tubule in a mammal, even when a concentrated urine is produced through the counter-current multiplier system (15).

CONCLUDING REMARKS

At the beginning of this presentation, I mentioned that the common laboratory animals are frequently studied, not necessarily because they are best suited for the experiment, but because it is possible to keep these animals in a healthy state in the laboratory. In widening the choice of experimental animals, the questions arise of obtaining, maintaining, and experimenting with nondomesticated animals. With respect to the invertebrates and lower vertebrates, this is usually not difficult. It is often quite easy to copy the normal habitat under laboratory conditions. With respect to the vertebrates and mammals, it is more involved.

I feel that it is important and desirable to study the animal in its natural habitat, before it is taken into the laboratory for further experimentation. The complex environmental and behavioral factors are not fully appreciated, if one is not familiar with the animal's natural habitat. In adapting nondomesticated animals for laboratory work, it is often best to start with immature animals because these can be trained and tamed. Tranquilizers can be used to advantage during the training period.

There is no doubt that the common laboratory animals will continue to retain their positions as the most widely used experimental subjects. I hope, through this discussion, however, to have pointed out that it is both advantageous and exciting to include some of the more unusual and more highly specialized animals in our studies.

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Control of quality in laboratory animals to increase effectiveness of biological research

JULES S. CASS

The Kettering Laboratory, Department of Preventive Medicine and Industrial Health, College of Medicine, University of Cincinnati, Cincinnati, Ohio

A PROBLEM CANNOT BE SOLVED RATIONALLY unless it is properly analyzed. I shall attempt to define the problem of quality and its control in laboratory animals, examine its component parts, and discuss the aspects that are in need of understanding and solution.

Failures in biologic experiments may have nothing to do with the soundness of a proposition or a hypothesis. They may result from the lack, or malfunction, of a particular technique, or of a particular adjustment or of some other factor. One of these other factors underlying some of the failures in biological and medical research is the laboratory animal.

A discussion of the following four questions will provide a basis for examining the problem of ineffective research due to the malfunction of laboratory animals: *a*) What is "quality" in laboratory animals? *b*) What need is there for improved "quality" in animals used in research? *c*) How has the quality of laboratory animals been improving? *d*) Once achieved, how may improved "quality" in laboratory animals be maintained and reinforced?

"Quality" may be defined as a characteristic constituting the basic nature of something, or may refer to the degree of its excellence. With these criteria in mind, let us consider what characteristics constitute improved quality in a laboratory animal. We should include in our considerations an anatomic, physiologic, and psychologic constitution freed of extreme variations; vigor in reproduction, growth, and other vital functions; a state of health that can withstand the "slings and arrows" of the laboratory environment; freedom from complicating spontaneous diseases; domestication and ability to adapt to changes in the environment, including the diet; living and reproducing in confinement; and the characteristics of tameness, ease of handling, even temperament; and intelligence, including the ability to be trained.

A desirable constitution is an aggregate expression of many factors, rather than an overall constitution that is resistant or susceptible to all responses. We may classify the characteristics that add up to improved quality in laboratory animals as those that are inherited; those that exist somatically; and those that are influenced by the laboratory environment, including man's manipulation.

WHAT NEED IS THERE FOR IMPROVED QUALITY IN ANIMALS USED IN RESEARCH?

Scientific literature is richly endowed with the far-reaching biologic and medical discoveries of the past. These outstanding results were achieved by using the quality of animals of yesterday. In addition, we have all striven to obtain and, in time, have settled upon laboratory animals that have performed satisfactorily, and, in our judgment, without complicating the outcome of our experiments. May we conclude, then, that the need for improved quality is not critical to more efficient research? We need only to remember that specifications for quality in animals change with newly acquired information and skill. The last decade, especially the last 5 years, has provided new information and skill pertaining to laboratory animals in phenomenal abundance.

The era of more strictly descriptive biologic research has moved gradually into one of increasing quantitation with the evolution of finely developed techniques, devices, and materials. The accurate and sensitive electronic instruments, the purity and precise definition of our chemical reagents, and the high degree of technical skill that exists in some areas of research using animals, have made the highly variable, and frequently unpredictable laboratory animals of yesterday, and some of today, archaic by comparison. More seriously, they are frequently the primary basis of poorly productive or inefficient research today.

In the planning of experiments, are we using the same judgment in deciding what the specifications for laboratory animals must be to meet the quality of our research as we use in the selection of instruments and reagents? Animals, analogous to instruments, can display inherently poor accuracy (great individual variability); poor sensitivity to a stimulus (a wide range of resistance or susceptibility); and can develop spontaneous defects that modify their accuracy and sensitivity. These modifiers are inherited defects, diseases due to microbial agents, parasites, neoplasms, malnutrition, environmental stresses, and aging.

The need is to reduce biological problems to simplest terms, a single cause-and-effect relationship. This is difficult to achieve in complex organisms, and, at best,

simplification generally includes several such relationships. Once fundamental relationships have been painstakingly explored in animal populations of uniformly good quality (good sensitivity and accuracy), the complicating factors (spontaneous and induced), can then be added intentionally to evaluate their effect in the system. The anatomist and the physiologist would want an animal with an uncomplicated anatomy. The pathologists would want to avoid a complicated morbid anatomy. The microbiologist, particularly the virologist, must guard against activating latent organisms in test animals, and so on from specialist to specialist.

Is it not peculiar that where lower forms of life are used, we have become very exacting, but that, as we ascend the phylogenetic tree, we demand less and less quality in the organisms that we use? With the lower forms of plant and animal life, we have recognized and accepted the concept of type colonies or cultures having defined characteristics and reared under prescribed conditions. Ample evidence exists to support the need for such precise selection and care of bacterial, protozoan, algal, and tissue and cell cultures, as well as colonies of drosophila and mosquitoes. Use of these types of cultures has become commonplace, almost exclusive in some research.

Some investigators have been compelled by the nature of their animal problems to take up the challenge to remove one by one the handicaps to effective and productive biological research. They have used the past as a guidepost, not as a hitching post, and have moved ahead. Others have evolved a philosophic logic over the years to justify and support their use of laboratory animals that are "normal as nature intended them." The truth is that the animals we are using are only as "normal" as we are able to produce and rear them, or as they are delivered to us by a commercial producer or dealer.

Each producer of laboratory animals selects an already complex system that nature provides and embroiders upon it by using in varying degree practices of sanitation that limit or extend the introduction and spread of diseases; practices of breeding that produce shifts in the genetic characteristics of a colony; practices of nutrition that range from improper to adequate, and practices of care that range from inadequate to outstanding. All of these in concert have a bearing on how great an adjustment these animals need to make when they are used in the laboratory.

What are some of the reasons offered for using animals "as they are" without the urgency to improve their quality? I shall present some statements that are commonly made by competent investigators and in rebuttal offer some bases for justifying our serious consideration of animals of quality.

Statement 1. I have used this strain, breed, or species of animal from one particular source throughout my work and it has proved satisfactory. I hesitate, and see no reason to change now.

Discussion. If this is so, the investigator has the answer to his animal needs. This statement, however, is particularly disarming. It frequently, though unintentionally, tells only part of the story. What does not appear in the statement are the trials and tribulations that were suffered by the investigator before locating the source of this animal that by comparison he found to be most satisfactory at the time. The memory of this sometimes traumatic experience may well give him pause to venture a comparison of the animals he selected yesterday with the animals of improving quality that can be supplied or produced today. The increasing indispensability that an investigator attaches to a particular colony of laboratory animals, as he derives more and more information from them, is so different from his effort and willingness to improve and modify techniques and methods that also have produced vast quantities of data.

Inefficiency due to malfunctioning animals can be subtle and too commonly is unmeasured. It can involve the death or illness of animals prior to experimentation, leaving behind for investigation a biased population of resistent survivors with some harboring latent infections, and morphologic and biochemical lesions of varying degrees and types. Frequently the user of this group of animals is unaware of this history.

Any concurrent loss of animals due to agents outside an experimental procedure, whether spectacular in number or not, requires that enough animals be added to an experiment to help mollify such a potential loss. It becomes evident that laboratory animals with fewer of these complications can be used in smaller numbers and increase the precision of biological work.

Statement 2. Animals of better quality are more expensive and less readily available.

Discussion. This infers that the initial cost per animal is much greater, or that the cost of caring for an animal of improved quality is more. Actually, the purchase price of the best quality of mice and rats available commercially today is only a trifle more than that of the average quality. Anyone who has kept records of the cost of "conditioning" animals, especially dogs, cats, and rabbits, finds any such savings in purchase price of animals of undefined quality quickly dissipated. Such "conditioning" involves ridding the animals of parasites and giving prophylactic drugs, or vaccines. It involves waiting through a period of isolation until the incubation period of common illnesses, taking its toll of animals, has passed, and then waiting until the survivors attain a reasonably good body weight and appear to be well. Animals of good quality are free of many of these problems, and can be conditioned more quickly—and inexpensively. The proper care for an outstanding group of laboratory animals is little, if any, more than it is for one of poor quality. When one considers the loss of time and effort of the investigator working with a malfunctioning animal, the proportional charges for the care of better animals are, in fact, less. Money expended in producing and caring for animals of improved quality becomes a form of insurance for the successful outcome of experiments.

How would you calculate the dollar-value of losses like the following quoted from published articles in reputable journals? "About 350 days after the first exposure, the entire rat colony developed a pulmonary infection, which resulted in a number of deaths in both the irradiated animals and the controls and was responsible for unusually rapid mortality at that time" (6). Some of these pulmonary infections such as viral bronchiectasis in the rats are contracted within days after birth, but do not become apparent until later in life. Another investigator reported that comparisons in reticulocyte counts could not be made between the experimental and the control animals because, "There is some evidence that at least part of the rat colony was infected with Bartonella. Such an infection would explain a high reticulocyte count. Under 'normal' (my own quotes) conditions a high reticulocyte count is the only sign of a Bartonella infection" (3). In an early attempt to adapt poliomyelitis virus to albino Swiss mice, signs appeared resembling those that, at first, were thought to be due to the poliomyelitus virus, but which were subsequently proved to be due to the activation of a latent lymphocytic choriomeningitic virus present in the colony (8). "Many of the treated dogs died of pneumonia. . . . Possibly many of the pound dogs used in these experiments had latent pulmonary infections" (7). What did such animals really cost the investigator?

Statement 3. Since each individual of a species varies so much in itself, even between serially repeated trials in the same animal, how can any control of quality in animals be expected to decrease such individual variation?

Discussion. The use of an animal as its own control often arises from a need to limit the variability and individuality of response within a group of unselected animals of a species. It is inferred that the wide variations are due for the most part to inherited and somatic characteristics. What we need, as mentioned above, are measurements to determine the role played by modifiers in the environment such as: prior medical history, state of health and nutrition, and also temperature, light, and humidity.

Statement 4. Much of the information resulting from experiments using animals is intended for use in understanding better the biology of man. Because the human population is heir to various and sundry diseases, defects, and aberrations; then, laboratory animals, in order that they may serve as biological models for finding solutions to biological problems in man, must also include in their population the diseases, defects, and aberrations to which they are heir. This school concludes that it is indefensible to eliminate from a colony of animals, to the ultimate, all deviates and animals showing abnormal characteristics and attempt to use the resultant population of animals to solve broad human problems.

Discussion. Are these investigators saying, for instance, that because the human population suffers a varying incidence of diseases X, Y, and Z in organ systems A, B, and C, that a more representative population of laboratory animal should also suffer a varying incidence of diseases in systems A, B, and C regardless of the fact that the diseases in the rats are caused by agents differing from (with incidences differing from) and with natural histories and pathogenesis differing from diseases X, Y, and Z in man?

The ultimate degree to which we may eliminate individuality, deviates, and abnormal characteristics from any population of laboratory animals is but a gleam in the eyes of geneticists. A perusal of Gruneberg's, "The Genetics of the Mouse" (5) provides more than adequate evidence to illustrate that modification of genetic patterns in these little animals by selection or by mutation is something like lifting the lid in Pandora's Box. An amazing array of deviating characteristics can and does fall out along with the selected characteristics that may be more uniform.

In defense of the view that individual variations in animal populations should not be minimized in all situations, consider the following: If one is using a mouse to test quantitatively for riboflavin, it would be advantageous to use animals that are as uniform in this characteristic as possible. Also the use of a uniform strain of animals would help simplify the investigation of how riboflavin functions in mammals generally. However, if one was investigating the riboflavin requirements of mice as representative of mammals generally, one might get a wholly erroneous picture by using animals with limited variability, such as inbred strains.

Statement 5. I am just doing some very preliminary work.

Discussion. This infers that the quality of the animal is relatively unimportant at the time and that these trials are likely to be an experience in the techniques involved. Large variability or poor reproducibility in preliminary tests many times influences the selection of methods and experimental design, and may also discourage the pursuance of an otherwise worth-while investigation. This investigator's attitude suggests little consideration regarding the possibility of extending the preliminary trials into an investigation of some length and precision in which one would want to use the same strain and source of laboratory animals throughout for purposes of good comparisons and reproducibility.

Can we afford to produce and house large numbers of animals so that those surviving or proved fit for use number enough to fill our requirements for animals in research? The increased cost to produce and to house animals and to provide good care, as well as the overall increased cost of research, imposes very compelling reasons for improving and controlling the quality of our laboratory animals.

HOW HAS THE QUALITY OF LABORATORY ANIMALS BEEN IMPROVING?

The quality of laboratory animals has been improving through a better understanding and selective manipula-

tion of the inheritable characteristics of animals and through the control and manipulation of the environment. Interplay between these two sets of factors is the determinant in the individual variation displayed by an organism. As investigators, we recognize the need to select "species" of animals that respond in some measurable way to experimental procedures. We also recognize the continuous need for colonies of animals whose genetic constitutions include great individual variation. Fewer of us have practiced the selection of reproducible genotypes within a particular species for use in experimentation.

Experiments must deal with individuals or small samples of a population, yet provide representative observations capable of application to whole populations. One useful means for separating and analyzing the factors that pertain to a given response is the control of individual variation in the expression of a particular characteristic within a strain, while permitting the full expression of variation between strains. Several methods for establishing such populations of laboratory animals have been devised, including selection with inbreeding (which involves 20 or more generations of brother to sister mating); and close breeding and the creation of homozygous strains through out-crossing. Individual variations expressed in a heterogeneous population have been used to create new inbred populations of animals expressing an individuality (a specifically selected characteristic or group of characteristics) for each strain, thus fixing such individuality. Such "fixed" individuality expressed by inbred strains has been used in mammalian genetics, in separating the factors that contribute to resistance to disease, in cancer research, in nutritional research, and in dental caries work. Various inbred strains have been suggested as hematologic test animals for biologic preparations because strain differences in blood constituents have been noted. Strain differences in chemical metabolism may serve to test drugs and offer possibilities for investigations on metabolic pathways.

The quality of laboratory animals is improving through the practice of selective genetic control and manipulation. Several specifically characterized inbred strains of mice are now available commercially in this country. The production of laboratory animals (inbred or not) by truly randomized mating has become another controllable practice to minimize the drift from an established genetic constitution in a colony.

A second outstanding means for improving quality has been the production of millions of mice and rats freed of specific microorganisms and internal and external parasites by Caesarian-derived colonization. Commercial producers are breeding such animals and making them available in numbers that should soon be able to meet the needs of research. Various laboratories and institutions in this country and abroad are producing such animals for their own use. The other common species of laboratory animals commercially available in substantial numbers have not yet begun to reach this quality, although various laboratories in the United States and abroad have found it well worth their time and effort to produce colonies of Caesarian-derived guinea pigs, dogs, and other species for their own research.

Breeding colonies of animals freed of many disease-causing agents are started with litters derived by Caesarian section from conventional mothers, and suckled on germfree mothers, or reared entirely as orphans on sterilized semisynthetic diets.

The notable difference between a conventional and a Caesarian-derived colony is not a difference in genetic manipulation, since both can be, and very frequently are, started with a limited sample of the gene pool from its original stock represented by a limited number of breeding pairs. In neither case would the precise genetic constitution be predictable. The difference is in the elimination of some microorganisms and macro-parasites from the Caesarian-derived stock. Any known disease-causing agents that do not pass through the maternal placenta to an embryo can theoretically be left behind when full-term fetuses are removed sterily in relatively inexpensive equipment and with a simply executed technique to an environment freed of these same agents (4).

All biological estimations must be carried out in the face of large error due to animal variation. Why, then, risk making the error still larger by using a more complicated organism than is necessary, and which can give rise to unforeseen and unaccountable deviations? Animals from Caesarian-derived colonies provide us with a less complicated and, therefore, more reliable organism for research.

In addition to the control of individual variability through genetic manipulation, and of variability due to disease through Caesarian-derived colonization, the quality of laboratory animals has been improving through the control of somatic and functional variation by standardization and availability of adequate diets; through the control of individual and group response; by more knowledgeable manipulation of the physical environment (ventilation, humidity, temperature, housing, light, and sound); and through the control of individual and group response by considered manipulation of the biological environment (selected animal grouping, population dynamics, vastly improved animal care procedures, and considerate handling and restraint) (1).

ONCE ACHIEVED, HOW MAY IMPROVED "QUALITY" IN LABORATORY ANIMALS BE MAINTAINED AND REINFORCED?

Quality control is a means of measuring the degree to which prescribed specifications are attained and maintained. Controls are established, depending upon the characteristics and quality desired.

If the characteristics to be controlled are genetically bound, breeding practices and breeding records must be rigidly set and kept. Even so, the problems of genetic drift and mutation lurk in each colony of animals.

Several quality control techniques are practiced to measure the maintenance of certain characteristics in inbred strains of animals. Isografting, the successful grafting of skin from one animal of an inbred strain to another of the same inbred strain, has been used to indicate continuing "sureness" of the strain. The uniform ability of an inbred strain to support growth of specific tumor transplants, the uniform degree of susceptibility to a specific pathogenic organism, and the uniform degree of incidence of a specific spontaneous tumor are all measurements for the control of quality.

If the quality to be maintained is related to freedom from certain disease agents, a breeding population must be sampled regularly to reassure the continuing absence of these agents. For example, mice are examined for the organisms of ectromelia and salmonellosis; and rats are studied for the incidence of pleuropneumonia-like organisms, or for the virus of enzootic bronchiectasis. Animals from a Caesarian-derived colony are susceptible to the common pathogens, and experience with placing these animals in the same room, but on separate racks with conventional animals, has demonstrated that they are not unusually susceptible, but will slowly become reinfected and reinfested when exposed under conditions of good sanitation. All mortalities and illnesses should be examined for cause. Examination and culturing retired animals in any breeding colony provide good measurements of the incidence of agents of chronic disease.

Once certain disease agents have been eliminated from a colony, there must be a sound, fully routinized program to keep them out. All new arrivals must be isolated. The care of the animals will require strict adherence to a standard routine of feeding, watering, sanitation, and a control of incoming personnel, equipment, and supplies. This is not a simple matter, but it is not formidable. The methods are known and practical (2). The results of a research effort will reflect this attention to animal needs.

Multiple small breeding rooms, as well as small experimental rooms with unit care and management, will increase efficiency of operation and effectiveness in the control of quality.

In summary, we can conclude that we now possess the "know-how" to produce laboratory animals of greatly improved quality for executing more effective biological and medical research. For example, we can minimize genetic "drift" in an established colony by practicing truly randomized mating. We can produce or obtain commercially inbred strains of mice that respond more accurately (lessened individual variability); more sensitively (narrowed range of responsiveness, resistance or susceptibility) within a particular strain; and that express a wide range of individual variability and sensitivity between and among strains. These useful qualities also have been developed through selection in a limited number of other species of mammals.

With increasing ease and with reasonable cost, we can produce or purchase from reliable commercial sources, animals freed of specific disease-causing agents which otherwise modify unpredictably the sensitivity and accuracy of our laboratory animals. The nutritional requirements for our common species of laboratory animals have been defined adequately so as to permit the feeding of standardized diets (natural, semisynthetic, and synthetic), which are milled commercially, or can be compounded by investigators from commercially obtainable ingredients. We are learning to recognize and to control increasing numbers of agents in the laboratory environment, such as light, humidity, and ventilation that modify the accuracy and sensitivity of response.

The continued and reinforced control of the greatly improved quality of laboratory animals by trained and practiced animal caretakers, animal technicians, and by informed investigators already has begun to reflect in the efficiency, reproducibility, and productivity of biological and medical research.

We cannot enjoy the luxury of observing one variable or characteristic in a living organism, while allowing others to change in what we hope is a random fashion. We have to discover all relevant variables and submit them to experimental control, whenever possible. I suggest that our determined use of laboratory animals of controlled quality will serve to bring closer this necessary goal.

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Laboratory animal caretaker training

L. R. CHRISTENSEN

Berg Institute, New York University Medical Center, New York City

THERE ARE AT LEAST FOUR IMPORTANT parameters in animal experimentation. First, the animals available for experimental use; second, the facilities for their care; third, the quality of the personnel who care for the animals; and, finally, the nature of the experiment itself. Other members of this Symposium have discussed various aspects of these parameters. It is my task to discuss the third, another aspect which can contribute to the quality of animal experimentation, increasing the knowledge and competence of the animal caretaker.

The constantly increasing use of experimental animals in the United States is awakening an interest in the proper care of these animals. This Symposium is a case in point. While exact information is not available on animal usage in the United States, I may not be far from right in suggesting that the United States uses more laboratory animals than the rest of the world combined. For instance, one commercial producer of laboratory mice within 30 miles of New York City produces more mice than are used for all purposes in Great Britain.

This awakening interest in animal care has led to the realization that proper care is essential in animal experimentation and that it is no longer a profession for unskilled workers; but, rather, that the intelligence and knowledge and skill of the animal caretaker must be equivalent to that of the other technical personnel on the research team. There is also increasing realization that the laboratory animal is a major source of uncontrolled variables in biological experimentation. Development of Caesarian derived animals, free of the common infectious diseases, has resulted in a re-evaluation of some traditional base lines in biological research. The realization that such factors as diet, temperature and humidity, cage sizes, amount of exercise, and other factors related to the well being of the experimental animal can significantly affect research has led to the upgrading of our animal facilities and to the realization of the need for better caretakers.

The broad subject of animal care personnel can be divided into three areas. One is concerned with the care of the animal while actually under experiment. Here the requirements of the investigator are paramount and his technical staff are directly concerned. Another area is concerned with animal care at the professional level. Dr. Clarkson has discussed training programs designed to provide this technical competence. I will discuss the third area, designed to increase the competence of the non-professional animal caretaker.

Any consideration of technical training for animal caretakers must necessarily refer to the British experience. In Great Britain, an Animal Technicians' Association was organized about 11 years ago. This group composed of animal caretakers has established training and certification plans administered by the Association and carried out with the cooperation of academic institutions and investigators in Great Britain. (*1*) Courses with regularly scheduled classes are held in the major cities. At intervals the Association holds examinations for the various levels of certification and issues certificates to those candidates successfully completing its examinations. At the present time, there are about 1,500–2,000 animal technicians in Great Britain, of whom about 40% hold at least one of the Association's certificates. A cursory inspection of employment notices in British scientific journals indicates that in many instances employment notices stipulate possession of an ATA certificate as a condition of employment. In Table 1 are shown the results of the various certifying examinations held in Great Britan over the past 10 years (personal communication).

The British system has attained one of the objectives of the United States program. Today a certified animal technician in Great Britain receives a salary practically equivalent to the salary of a laboratory technician, and his prestige in the scientific community is equal to that of the laboratory technician's. In fact, I am told that not infrequently a laboratory technician will transfer to the animal technician field because of his interest in animals and because he suffers no loss of salary or prestige. This is certainly not the case in the United States at the present time.

For a number of years the Institute of Laboratory Animal Resources and The Animal Care Panel have had education committees studying and promoting the education of animal caretakers. A number of courses for training in laboratory animal care have been offered, both within and outside the ILAR-Animal Care Panel programs.

Among these are the two nation-wide courses for animal caretakers given at Berg Institute, courses at Roswell Park, and those given by Dr. Bennett Cohen at the University of California at Los Angeles. The philos-

TABLE I

	Examinations	Candidates	Passed
Preliminary exams	6	132	108
Associate exams	14	236	144
Fellowship exams	3	25	9

ophy of these courses has been that the mechanics of animal care can be taught on the job but that the basic biological background essential for proper understanding of laboratory animals and their care should be provided by these courses. The majority of these lectures are given by animal supervisors, veterinarians, and other scientists on such topics as anatomy, physiology, genetics and breeding, sanitation and hygiene, animal disease and its control, and similar subjects related to animal care. In all cases emphasis is placed on the relation of the subject matter to animal care and its practices.

It soon became obvious that courses in different localities differed in content of instruction and aim. The education committees of the ILAR and the Animal Care Panel, therefore, recommended the establishment of a certification program for animal technicians. Such a group, the Animal Technicians' Certifying Board, was established last year by action of the Board of Directors of the Animal Care Panel. The board has the following objectives: *a*) To establish levels of competence of animal care; *b*) to establish and conduct certifying examinations for animal technicians; *c*) to issue certificates to qualified candidates in the name of the Animal Care Panel; and *d*) to aid in the establishment of training programs designed to prepare candidates for the certifying examination.

Detailed qualifications have not been prepared for all categories, but the Board has established the following three levels of competence: Junior Animal Caretaker, Senior Animal Caretaker, and Supervisor.

In collaboration with a number of technicians and supervisors, it was decided that a Junior Caretaker should possess a knowledge of the following subjects:

1) Feeding and watering of animals; cleaning of cages and equipment.

2) Animal handling and its tools.

3) General and personal safety in the animal house.

4) Personal and animal hygiene.

5) Elementary scientific vocabulary.

6) Basic biology (anatomy and physiology).

7) Recognition of the clinical signs of common diseases of animals.

8) Animal sexing, gestation periods, and weaning ages.

9) Simple record keeping and animal identification methods.

10) Basic principles of experimental design.

11) Sanitation and disinfection.

12) Pest recognition and control.

13) Humane euthanasia.

14) The caretaker's responsibility to animals and to investigators.

The Senior Animal Caretaker should be expected to possess a more extensive knowledge in all of the above points and, in addition, knowledge in the following areas:

1) Basic work plans and work schedules.

2) Administration of drugs under supervision.

3) Elementary laboratory procedures (fecal examinations and gross autopsies).

4) Animal nutrition, including the preparation of special diets.

5) Elementary genetics and animal breeding.

6) Elementary surgical procedures and bleeding.

7) Record keeping and administrative procedures.

Supervisor is the highest certificate offered. These are for individuals who can carry on the day-to-day operation of an animal house, under the policies established by the professional director. Their knowledge in all of these fields should be so extensive that they can teach the subjects to Junior and Senior Caretakers. In addition they should be competent in the following:

1) Purchasing of animals, supplies, and equipment for the animal house.

2) Laboratory procedures, including parasitological examinations, necropsy of animals, and procurement of tissues for histopathology, and elementary bacteriological culture techniques.

3) Surgical procedures, including preparation of surgical equipment, supplies and apparatus, and sufficient knowledge to act as a surgical assistant.

4) Administration in the animal house, including bookkeeping and cost accounting.

5) Fundamentals of cage design and facility design and construction.

6) Experience in research projects.

A number of courses are now in progress or have just been completed designed to prepare Junior Technician candidates for the certifying examination. In the majority of instances, these courses have been sponsored by local branches of the Animal Care Panel. Courses have been or are being given by the Metropolitan, New York, branch; the Madison, Wisconsin, branch; the Philadelphia branch; the Southern California and the Northern California branches; and are planned soon for the Boston branch. In addition to these courses sponsored by local branches, courses for Animal Technician Training have been given in Washington, D.C., and at Roswell Park in Buffalo, N.Y.

The Metropolitan New York branch has established an educational committee to whom inquiries for information on courses, course outlines, and examination for Junior Technician can be addressed. The chairman of the committee is Alfred Havemeier, Berg Institute, N.Y.U. Medical Center, 550 First Avenue, New York 16, N.Y.

At the international level, the International Committee for Laboratory Animals (Dr. Lane-Petter, Laboratory Animals Centre, M.R.C. Laboratories, Woodmansterne Road, Carshalton, Surrey, England) has prepared outlines of courses for animal caretakers.

There is one major difficulty with the program outlined. Emphasis is placed on a well-rounded education in all aspects of animal care. In some institutions, however, and particularly in commercial breeding institutions, there are highly competent caretakers who have no experience with species other than the one being bred, and who have no opportunity to obtain such experience. While this difficulty is more apparent than real at the level of Junior and Senior Technicians, it does become significant at the Supervisor level, because the latter is an individual qualified to operate a large animal facility with numerous species. The Certifying Board is aware of this difficulty and is attempting to find a solution.

In summary, an integrated program now exists here for training animal caretakers. A certifying program has been established, under the auspices of the Animal Care Panel, and courses are being offered to prepare candidates for certification. These programs should result in the recognition of animal care as a desirable career and should afford a means to significantly improve our standards of care within the next few years.

For those interested in training courses for caretakers, I would suggest that they contact the nearest local branch of the Animal Care Panel or the secretary of the National organization, Dr. Robert J. Flynn, Division of Biological and Medical Research, Argonne National Laboratory, P.O. Box 299, Lemont, Ill. Inquiries regarding the certification program can be addressed to Dr. L. R. Christensen, chairman, Animal Care Panel, Technicians' Certifying Board, Berg Institute, N.Y.U. Medical Center, 550 First Avenue, New York 16, N.Y.

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Graduate and professional training in laboratory animal medicine

THOMAS B. CLARKSON

Bowman Gray School of Medicine of Wake Forest College, Winston-Salem, North Carolina

THE EVOLUTION OF LABORATORY ANIMAL MEDICINE in the United States was recently reviewed by Cohen (1). It is of interest to note that laboratory animal medicine is perhaps the newest of the areas of specialization in veterinary medicine. The development of laboratory animal medicine in the United States began in 1850 and increased impetus in its growth began about 1945, when a significant number of medical schools and research laboratories employed veterinarians. In Chicago, for example, the University of Chicago, Northwestern University, the University of Illinois, and the Hektoen Institute for Medical Research employed veterinarians at this time. It soon became apparent that the veterinarian contributes significantly to the solution of many problems in the realm of laboratory animal biology.

Since that time, the concept of laboratory animal care has become one that is much more complex than merely "caring for the animals" (2). The purpose of this paper is to describe the current status of graduate and professional training in laboratory animal medicine.

The objective of currently available training programs in laboratory animal medicine is to provide individuals capable of establishing laboratory animal medical units primarily in medical school situations. In order to describe their training, it is necessary that we define the role of the laboratory animal veterinarian in an academic institution. The most important roles of the veterinarian are:

1) To represent veterinary medicine as a discipline in the academic community. The activities in this area involve consultations with research investigators on the selection of the proper animal species for a given experiment, advice on the spontaneous diseases of the particular species that might complicate results, suggestions for inducing various disease states, and aid with the interpretation of experimental animal data.

2) To establish and maintain standards of husbandry services. These activities involve training of husbandry personnel, control and standardization of laboratory animal diets, use of proper bedding materials, establishment of efficient and accurate breeding methods, provision for adequate physical environment and maximum standards of sanitation.

3) To establish and maintain those clinical services that are needed to expedite animal experimentation in the institution. This area involves examination, immunization and treatment of incoming animals and those being held on long-term experiments, maintenance of animal blood banks, care of animals in the postoperative period, and necropsy services.

4) To serve as an administrator for the unit. This concerns maintaining files on sources of animals, procurement of animals, and assignment of space for their housing, control of budgetary matters, and personnel management.

5) To participate and encourage research in laboratory animal medicine. This might well include a search for laboratory animal diseases resembling human disease, problems that might be used as experimental tools, or experimental programs designed to elucidate the physiological reactions of the laboratory animal to its environment.

6) To offer instruction in techniques of animal experimentation. If the experimental animal unit is to be a part of an academic institution, it is important that the veterinarian be competent in the techniques and philosophy of teaching. This instruction might take the form of a formal graduate school course, or be quite informal and involve the instruction of individual technicians or research investigators.

The training program at the Bowman Gray School of Medicine is one of increasing responsibility under supervision. Fellows for this program are chosen from graduates of schools of veterinary medicine on the basis of *a*) past academic record; *b*) interest in research and an academic career; *c*) aptitude for laboratory animal medicine; and *d*) ability to integrate himself for group work. Each Fellow is appointed for a period of 2 years.

During the 2 years of training, the Fellow spends about one-half of his time in the vivarium and the remainder is divided among four academic departments of the Medical School. During his half time in the vivarium, he is given experience in the medical treatment of all species of laboratory animals, the operation of a postoperative recovery room, the operation of a quarantine receiving room, the operation of a necropsy laboratory, the operation of a canine and ovine blood

bank, and the problems of vivarium administration. Regularly scheduled conferences are held in which the special anatomy and physiology of the laboratory animal is discussed. Spontaneous diseases of laboratory animals are reviewed, and methods for the production of experimentally induced diseases are discussed in detail.

Approximately half of the Fellow's time during the first year of training is spent in the experimental animal surgery laboratories of the Department of Surgery. During this time, the trainee is expected to gain advanced knowledge of surgical techniques, an appreciation for surgical research, and a more thorough understanding of the care of the animal during the immediate postoperative period.

During the next 6 months, he spends nearly full time in the necropsy laboratory. Gross and microscopic studies are done on all animals dying in the vivarium and, in addition, exercises are done in experimental pathology.

During the last 6 months, the Fellow spends approximately half of his time participating in the research activities of the Departments of Physiology and Microbiology. The object here is to acquaint him with the techniques and concepts of research in these areas.

In addition to this formal training, he is encouraged to do research on a problem of his own choosing in collaboration with a member of the faculty who, the program director feels, will give the Fellow good insight into the techniques and philosophy of research.

The training program in laboratory animal medicine at the University of California at Los Angeles, under the direction of Dr. Bennett J. Cohen, resembles the Bowman Gray program in many respects. The U.C.L.A. program places somewhat more emphasis on research and somewhat less emphasis on clinical laboratory animal medicine. In this program, the trainees spend 2 years in training. Fifty per cent of their time is spent in research and course work and the rest in professional apprentice training. This program offers a fine opportunity not only to train academically oriented veterinarians in research, but also to develop an integrated program in environmental physiology, with specific reference to the laboratory animal environment. A wide range of courses are available at U.C.L.A. for the didactic instruction of these Fellows. Courses are chosen jointly by the trainee and the program director. In addition to formal courses, seminars are held at least twice weekly to discuss problems in the field.

Professional apprentice training at U.C.L.A. involves postoperative care of laboratory animals, maintenance of canine blood banks, breeding of animals, training of animal care technicians, necropsy laboratory procedures, and discussions of the design of animal quarters.

In summary, we should emphasize that it is important that the laboratory animal veterinarian be trained to organize independently a program of teaching, research, and animal care. Post-doctoral programs for training such individuals are now available at the Bowman Gray School of Medicine of Wake Forest College and the University of California at Los Angeles.

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Housing for research dogs

N. R. BREWER

University of Chicago, Chicago, and Argonne National Laboratories, Argonne, Illinois

CRITICAL STUDIES ARE NEEDED offering good evidence as to what constitutes optimum conditions of housing for the purposes for which most dogs are kept in research laboratories. The literature is barren of information based on such studies, and there is probably more disagreement as to what represents optimum facilities and care for the dog than exists for any other laboratory animal.

Most of the disagreement centers around the question of need for out-of-cage exercise. Is it good management to house dogs in cages for long periods of time? Is the construction of individual pens and runs justifiable, especially in an urban area? Should outdoor runs be recommended? Should dogs be housed in groups in pens?

Because there is so little in the literature, I tried to obtain some information through the medium of a questionnaire. They were sent to the physiology and surgery departments of 98 medical schools in this country and in Canada, and to the membership of the American Board of Laboratory Animal Medicine. Replies were received from 89 of the 98 medical schools, a 91% return. From some institutions three replies were received. From other institutions only one reply was received with the advice that it was representative of the whole institution. Because of this, the replies were assembled and treated as though each institution answered but once.

The replies indicated that 17 institutions used only cages, that four used only pens, and that 68 used both. The questionnaire asked for a listing of the advantages or disadvantages of cages and pens. The semantics used in the replies was not uniform, but there was general agreement.

The main advantages cited for housing in pens were: *1*) More space is allowed for exercise; *2*) the dogs seem more calm, and bark less (when housed in groups); *3*) the animals and the quarters can be kept cleaner at less cost; *4*) there seems to be a lower incidence of skin disorders; and, *5*) dogs are in better condition for operative procedure.

Some of the comments stressing the advantages of maintaining dogs in pens are presented:

"Twenty-four hours after surgery, dogs placed in pens recover from surgery with less difficulty."

"We find these dogs do better over in the holding area where they have sunlight, air, adequate runs [than they do in cages] and the general well-being of the dog seems to be better under these circumstances."

"We have found that collies, in particular, lose weight and generally do poorly in cages, but do well in runs. Most of our postoperative feeding problems can be corrected if allowed an exercise period daily before feeding. In some of our cardiac studies, survival after the first 24 hours was correlated with out-of-cage exercise. Many of our long-term caged dogs develop atrophy of the muscles of the hind legs and difficulty in walking. Many long-term caged dogs develop abnormal behavior traits which can be reduced by daily exercise periods. This is especially true of puppies. Weight gains in many operated dogs can be correlated with daily exercise."

"If we had to choose between pens and cages for long-term animal care . . . our preference would be for individual pens. . . . Cages are inadequate for chronic experiments. We have found deterioration of animals maintained in cages for several months."

"Dogs in cages are less normal, less healthy, and less happy than dogs who have freedom of movement. While it is possible for caged animals to be let out for exercise by a caretaker, it is simpler and surer to let the dogs exercise themselves."

"A colleague studying muscle power found caged animals unsuitable because the loss of muscle power in the chronically caged animal obscured the possible drug effect being studied."

"An area of 10 by 12 ft can accommodate up to 10 dogs (depending on size), which would make 12 sq ft per dog. Most dogs are gregarious and remain healthier and happier in such quarters. They do very little barking compared with individually isolated animals and therefore debarking is not required even when in proximity to a hospital. It is necessary that good identification procedures be followed, it is best that the sexes be separated, and the occasional fighter must be watched for . . . ."

"It is our feeling that many factors tend to produce atelectasis in the . . . dog, [and that] the amount of exercise that it has before the study is [possibly] an important factor. In some work on experimental pulmonary edema we noted on microscopic examination that there was more atelectasis than could be accounted for by the experimental procedure. When we were able

to exercise the dog for a day or two preceding the experiment, it was our impression that the atelectasis did not occur or was minor."

The main advantages cited for the cage-housing of dogs are: *1*) Isolation is a necessity for many experimental procedures, such as metabolism studies; *2*) there is a conservation of space and, therefore, more economical housing; *3*) animals are protected from injury, or even death, from other dogs; *4*) the control of parasitism and of infectious diseases is made less difficult; *5*) feeding, handling, and control are simplified; weaker dogs obtain food they might not have obtained in competition; and, *6*) it is easier to identify and keep track of the animals.

Some of the comments supporting the advantages of housing dogs in cages are presented:

"The animals eat and grow better when they can receive personal attention from an attendant who cleans the individual cages and feeds the animals daily. The animals themselves are better adjusted in individual cages."

"We have had long experience in the housing of dogs in cages and some with animals in runways on a farm . . . if dogs must be confined at all, they fare much better in cages than in runways. . . . The choice between the two . . . is primarily a matter of the psychological state of the viewer."

"The animals that we have maintained in cages alone for periods as long as 3–4 years have remained apparently healthy and contented with no change in their disposition."

". . . dogs are kept in pens but . . . we do not have enough pens to keep [each dog in a separate pen]. In the plans for a new dog kennel, arrangements are made for individual cages for all dogs."

". . . dogs confined to cages for many years appear to be healthy, both physically and psychologically. When released from the cages for brief periods of examination and exercise, they appear to be unchanged in their attitude to the handlers and other personnel."

"The well-being of animals is influenced very little by the method of housing, but is dependent more upon the care given the animal. . . . For purposes of research, housing in cages is much more preferable."

"Dogs in cages for years seem to maintain good health without necessarily getting periodic running exercise. I have the untested impression, however, that caged dogs do better if allowed to run briefly daily."

"We have had individual cages for dogs for a period of 10 years and up to the present time have experienced no difficulty. . . . We do have a runway . . . for . . . exercising the dogs. . . ."

". . . The healthiest dogs I have ever seen had lived for over 10 years in metabolism cages. We recently scrapped a set of runway pens due to servicing problems. . . ."

". . . Research animals are too precious to be housed except individually, and we have had no difficulties on this score."

"I have dogs that have lived a cage life for 9 years and are in perfect condition."

"Frequently the type of experimental work to be done requires isolation and cages. Only rarely does it require social grouping of dogs . . . and pens. The animals are equally healthy and happy in either environment."

There is no agreement, even among those who have worked with dogs for years, regarding proper housing for dogs. There are very strong opinions for and against caging. I have the untested impression that the construction of the cage, run, or pen is more important to good management than whether or not it is a cage, run, or pen. It should be of adequate size for the animal, and of such design that the animal caretaker can spend a greater proportion of his time caring for the animal than in policing the quarters.

The untested impression that dogs in small compatible groups in pens are better prepared for many experimental procedures than are dogs in cages or in individual pens has some support from work with other species. Allee (1) has accumulated evidence showing that animals, whether they be protozoa, insects, fish, mice, or bison, have an optimum population density that is most favorable for growth, reproduction, or wound healing, and that this is usually somewhat higher than the theoretical minimum of two. It would be of value to determine the optimum population density per 100 sq ft for dogs in the research laboratory.

There are a significant number of qualified scientists who have testified that dogs have lived for years in cages, and have remained in apparent perfect health. Their testimony cannot be ignored. Such cumulative widespread experience is strong evidence that dogs can do well in cages. But that is not to say that any one dog could remain in good health if it had to spend long periods of time within the confines of a cage. Some dogs are able to accept confinement and restraint calmly, while others do not have this ability. According to DuBois (3) there is a difference in this respect among breeds, within breed populations, and even among littermates. However, the inability of some dogs to accept cage confinement tends to disappear and can be prevented by daily out-of-cage exercise periods.

Exercise, or lack of exercise, does affect many body tissues (2, 4). But the relative importance of exercise, even to dogs that are able to accept confinement, is not known. There are many untested impressions that exercise-conditioned dogs are better able to survive operative procedures.

For best management practices, it is probable that both pens and cages should be provided in a good animal quarters facility in a medical school or a teaching hospital. The optimum relative numbers and sizes of these units would depend on the answers to many questions that research should provide.

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Space requirements in the design of facilities for the small animal species

W. T. S. THORP

College of Veterinary Medicine, University of Minnesota, St. Paul, Minnesota

WHEN I SENT THE OUTLINE of this paper to Dr. Cohen, I became intrigued with the idea that apparently many words have been written and much has been said on the subject of space for small laboratory animals. In all apology to the intent of this Symposium, I am going to deviate a bit by not going very deeply into what I think, or others may think, regarding the specificities of necessary space for small laboratory animals.

We who are interested in the care of laboratory animals are gratified that the basic tool of much biological research—the laboratory animal—is now receiving due consideration by those whose investigations depend upon their use.

It is unfortunate that this type of discussion is, in reality, a rare thing and that the impetus to promulgate such a Symposium is due, I fear, to the adverse stimulus of the threat to the freedom of biological research.

DISCUSSION

The planning and operation of animal facilities is governed by many factors. Although one may have worked with and observed many of the animal facilities and space used for the small experimental animals, to make a recommendation on the space requirements for all of these species that would fit all situations, in all institutions, would be impossible and impractical. Any suggestions made should be considered as guidelines, and should be modified to suit the local needs. As many of you know, the entire planning and problem of necessary space for experimental animals usually resolves itself into a compromise and adjustment to the desires of the various interested research groups.

The average experimental animal room for the smaller species ranges from 200–300 sq ft. The design of such facilities brings itself down to the purpose of the experiment which requires these animals. The number of mice per cage, as an example. Are there to be a large number to a cage, or one or two? Are they to be used for infectious disease work? This makes a considerable difference in the planning of necessary space. There are no figures available to my knowledge, other than those based on a study made some time ago at the National Institutes of Health (*1*). These estimates should, perhaps, be re-evaluated in the light of newer methods and developments.

Estimated Net Square Feet of Experimental Animal Room Space*

Mice	7 per sq ft
Guinea pigs	2 per sq ft
Rats	5 per sq ft
Rabbits	1 per 2 sq ft

Estimates for Anticipated Production from a Breeding Colony

Mice	At rate of 50 per net sq ft annually
Guinea pigs	At rate of 2 per net sq ft annually
Rats	At rate of 1 per net sq ft annually
Rabbits	At rate of 10 per net sq ft annually

* Net space does not include corridors, vestibules, and storage.

The room size, if possible, should be limited to the animal capacity that one attendant can routinely take care of. This procedure will vary considerably in all research laboratories. It is best, if at all possible, to house one species, or strain, in a room. Rooms should be arranged off a corridor in such a way that traffic of materials and personnel should not have to pass through one animal room to gain access to another. Quarantine rooms for new shipments of animals will minimize the possibility of introducing any new infection into a colony.

The shape of an animal room relative to the space between racks and cages is, again, dependent upon the use. If it is primarily a production colony requiring the usual care and record keeping, less space is necessary. If it is an experimental colony, and there is considerable record keeping, treating, securing blood and examination of animals, then it will be necessary to provide more space as a working area and, in many instances, special facilities and equipment.

The square room has been used for production, as well as a long narrow room. Both appear to be equally satisfactory. If the racks are fixed to the wall, then it is important that the room be built in such a way as to obtain maximum wall space available for racks and shelves; thus, favoring the long narrow room. If the cages are on racks, and are removed periodically for cleaning, then enough space should be allowed for the traffic of material and equipment. Usually buildings are

built to a module, or bay, of a certain size, and, it has been my observation that in most instances in newer facilities, these rooms work out satisfactorily as animal rooms if properly equipped and air conditioned.

The location of the animal facility in the building will make some difference in the size and space necessary for actual housing. Housing of animals in a separate facility for an entire institution will not make it necessary to provide as much space for feed, equipment, and other supplies immediately adjacent to the animal room. Scattering of animal rooms throughout an institution by departments will increase the auxiliary, or supporting space, relative to the actual caging of the small laboratory animals.

One can observe new and prime facilities in which we would expect to find the best type of operation; however, the total facility and how it is used are necessary for good animals. There are some old, rather inefficient, and inadequate animal quarters used successfully for laboratory animals, providing there is an appreciation of the intelligent care needed for the particular colony. There are others where it is impossible to do this, because there is lack of personnel to care for the animals.

It is true that tile walls or similar material may provide some advantages but there are many fine laboratory facilities with only painted cement block walls. Tile construction is needed in the dog and monkey area. I sometimes get the impression that some very fine animal facilities have been built recently in this country that may create a false sense of security by the nonprofessional responsible for the well being of the laboratory animals. This is somewhat comparable to the individual who vaccinates his chickens or pigs, or treats them with a medicine, and, having done so, feels this is adequate, and becomes lax in observing basic operations for the prevention of disease which may affect the experiment.

That one has designed these facilities along certain plans does not eliminate the fact that he should go a step further and not forget the most important procedures of good animal management, including basic day-to-day care of the colony. The point that is being made, since we are discussing space for small laboratory animals, is that spacewise, you cannot design out the disease. These diseases may affect the outcome of whatever investigation is undertaken.

The number of animal rooms and the various species to be housed, will, of course, determine space requirements. If the facility plans to use primarily rabbits, larger areas should be placed in a given animal room; if it is predominantly mice, then smaller units may be arranged. This may vary some between the 2 for the other species, such as rats, guinea pigs, and hamsters. Here again, it depends a great deal on the use to be made of the experimental animals. The extent of the operation will determine the amount of storage space for feed and bedding, which should be separate. There is also the matter of adequate refuse disposal, as a result of routine cage cleaning. This depends entirely upon the situation in the institution. The volume of material has a great deal to do with the decision of having separate incineration facilities, or whether material will be sent to the central refuse disposal unit of the institution.

Although it involves non-animal or auxiliary space in regard to the total building, one would be remiss if the matter of air conditioning for small experimental animals was not discussed. The matter of air conditioning will have some bearing on the shape and layout of the room, particularly the inlets and outlets in relation to the cage racks. The air changes necessary are considerably more than those ordinarily used in comfort air conditioning for offices and households. There is also the matter of taking into consideration the amount of body heat given off by a large number of small animals in a given animal room. The system should, therefore, be amenable to adjustment, so that a constant temperature and humidity can be maintained.

There are a number of problems facing anyone planning work to give us more accurate answers on space requirements for laboratory animals. The figures referred to previously, based on experience of the National Institutes of Health, were made some time ago. I believe they should be reworked, and perhaps a study made at other institutions which have a somewhat different type of operation. There needs to be an evaluation of the air changes necessary for the various species, the humidity requirements, and the optimum and minimum needed by the various species of animals.

SUMMARY

Requirements for optimum quartering, or space, to house small experimental animals are not as well known as we might think or like to believe. This is true even by those primarily concerned in this area, either by trial and error, or by printed material.

We need critical research and investigation into correct and modern space requirements for small laboratory animals. We also need critical studies on air conditioning requirements.

Unfortunately, the best planning and plush materials are too often nullified by lack of financial support to provide proper care to keep pace with facilities. The animals, in a sense, have a lovely hotel, but in lacking proper care, they have inadequate maid and room service.

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LIPID METABOLISM

Biosynthesis and metabolism of unsaturated fatty acids[1]

K. BLOCH, P. BARONOWSKY, H. GOLDFINE, W. J. LENNARZ, R. LIGHT, A. T. NORRIS, AND G. SCHEUERBRANDT

Department of Chemistry, Harvard University, Cambridge, Massachusetts

RECENT ENZYMATIC STUDIES on the synthesis of fatty acids have thrown much light upon the intimate mechanism which produces extended aliphatic chains from smaller units (1, 2). In the purified soluble systems that have been described, the end products of condensation are the long-chain, fully saturated acids, among which palmitate usually predominates. The origin of the unsaturated acids has received much less attention and is usually treated as a separate problem. It has been thought for some time that oleic and other olefinic acids are formed secondarily from their saturated counterparts and not by an independent synthetic pathway. Initially, this view was based on indirect nutritional evidence (3) and later on the more conclusive experiments of Schoenheimer and Rittenberg demonstrating the conversion of deuterated stearic acid to oleic acid in animal tissues (4).

There have been numerous attempts to elucidate the mechanism of this conversion in vitro but they have not given any clear-cut results. For example, the claims that methylene blue can serve as the hydrogen acceptor in the desaturation of stearate, and that certain purines are cofactors in the reaction, [for a discussion see (5)] have not been well documented and the significance of these earlier studies remains obscure.

Perhaps the most important observation relating to the biosynthesis of unsaturated fatty acids was made by Andreasen and Stier in their work on the anaerobic nutrition of yeast (6). These investigators were able to show that under strictly anaerobic conditions, yeast becomes dependent on two types of lipids as growth factors. One is ergosterol, or a related sterol, and the second is an unsaturated fatty acid, such as oleate or linoleate. Since it is known that molecular oxygen is the obligatory electron acceptor in several intermediate steps of sterol biosynthesis (7), of which the cyclization of squalene to lanosterol is the first (8), it is clear why under anaerobic conditions ergosterol synthesis is blocked and why the sterol becomes an essential nutrient. On the other hand, the requirement of anaerobic yeast for an unsaturated fatty acid was an unexpected result. It suggested that the conversion of stearate to oleate does not occur by dehydrogenation but by a process involving molecular oxygen directly.

In extension of the findings of Andreasen and Stier, we have observed that resting yeast, when kept anaerobically, synthesizes saturated fatty acids exclusively, whereas in air, saturated and unsaturated acids are formed from acetate in approximately equal amounts (9). It was found next that whole cells or cell-free extracts of yeast transform palmitate or stearate into olefinic acids and thus catalyze the same type of reaction previously found in intact animals. A cell-free system that catalyzes the desaturation of palmitate or stearate and is obtainable from Hughes press extracts of yeast exhibits the following properties (9): Enzyme activity is particle-bound, the CoA esters of the fatty acids are the substrates, and both molecular oxygen and TPNH are essential for the reaction. The artificial electron acceptors methylene blue or phenazine methosulfate do not substitute for oxygen and, therefore, a flavoprotein-linked dehydrogenase is unlikely to be involved. On the other hand, the simultaneous requirement for oxygen and TPNH and also the insensitivity of the reaction to cyanide are recognized properties of the so-called oxygenase reactions which introduce hydroxyl groups into a variety of aliphatic and aromatic compounds. It, therefore, became attractive to visualize the desaturation of long-chain fatty acids as occurring in two steps, first a hydroxylation of the saturated acid to the 9- or 10-hydroxy derivative, and, secondly, a dehydration of the oxidized product to an olefin. The two isomeric hydroxystearic acids were synthesized and did, in fact, support the growth of anaerobic yeast as effectively as oleic acid itself (10).

However, subsequent experiments with labeled com-

[1] This work has been supported by grants-in-aid from the Public Health Service, the National Science Foundation, the Life Insurance Medical Research Fund, the Nutrition Fund, and from the Eugene P. Higgins Trust Fund of Harvard University.

pounds led to the unexpected finding that the hydroxy acids, though active in the growth test, were not metabolized noticeably. Only negligible amounts of the incorporated radioactivity could be recovered in the olefinic acids of the cell lipids, substantially all of the radioactivity remaining in the form of free or combined hydroxy acid. Moreover, the cell-free enzyme system of yeast, which acts on stearyl or palmityl CoA, fails also to convert the CoA esters of the hydroxystearic acids into olefines. These negative results make it difficult to retain the view that the hydroxy compounds themselves are precursors of unsaturated acids. Nevertheless, the requirement of the reaction for O_2 and TPNH makes it likely that the fatty acid substrate is in some way oxygenated in the course of desaturation.

In several instances, olefins have been shown to arise by elimination of phosphate ester groupings rather than by dehydration (11–13). In order to examine the possibility that a derivative of this type is an intermediate in oleic acid synthesis, we have synthesized the phosphate ester of 10-hydroxy stearic acid. This derivative also satisfies the fatty acid requirement of anaerobic yeast, but since no conversion to oleate could be observed, the effect is probably due to hydrolysis of the phosphate ester to the free hydroxy acid. Apart from the requirement for O_2 and TPNH in the synthesis of oleic and palmitoleic acids, the natural occurrence of certain long-chain hydroxy fatty acids points to the existence of a pathway involving oxygenated intermediates. In this respect, the recent isolation of 10-hydroxystearate from fecal lipids by James et al. (14) is of particular interest. These investigators have, moreover, shown that 10-hydroxystearate is formed from stearate by fecal microorganisms and apparently converted further to oleate (15). Whether the free hydroxy acid accumulating in this system is a true intermediate or a secondary product arising in a side reaction from an active oxygenated intermediate is, however, not clear.

Whatever the role of the hydroxy acids in the biosynthesis of olefins, the fact that they support the growth of anaerobic yeast without conversion to an unsaturated acid is an interesting phenomenon by itself. Since yeast does not synthesize olefinic acids in the absence of oxygen, the lipids formed under these conditions contain chiefly

TABLE 1. *Relative Concentrations of Fatty Acids In Aerobic and Anaerobic Yeast*

Acid	Growth Conditions	
	Aerobic	Anaerobic
C_{16}	5.1	56.2
C_{18}	0.7	22.8
C'_{16}	59.0	7.0
C'_{18}	35.2	14.0

Aliquots of the extractable yeast lipids were saponified and the methylesters of the fatty acids separated by vapor phase chromatography. The total lipid content of anaerobic yeast is approximately one third that of aerobic yeast.

TABLE 2. *Effect of Fatty Acids on the Anaerobic Growth of Yeast*

None	44	Stearic	43
Oleic	382	Elaidic	45
9-Hydroxystearic	390	D,L 10-Methylstearic	48
10-Hydroxystearic*	381	9,10 Epoxystearic	51
12-Hydroxystearic	222	9,10 Dihydroxystearic	0
12-Ketostearic	162		
Lactobacillic	152		

The data are expressed as numbers of cells in an aliquot of yeast suspension counted in a hemocytometer. Yeast was grown anaerobically on the medium of Andreasen and Stier (6) supplemented with ergosterol. * Isolated from natural sources and kindly provided by Dr. A. T. James.

saturated acids, some hydroxy acid, and unsaturated acids in very much smaller amounts than cells grown in air (Table 1). There is considerable evidence to suggest that monounsaturated fatty acids occur ubiquitously and are indispensable constituents of the cell, yet our results imply that the double bond per se is not a required structural feature for biological activity of the fatty acid molecule, at least for anaerobic yeast. In exploring this effect further, we have found that the growth of anaerobic yeast is supported not only by 9- and 10-hydroxystearic acids but also by 12-hydroxystearic acid, by 12-keto stearic acid, and by lactobacillic acid. Elaidic acid, 10-methyl stearic acid (2), 9,10-epoxystearic acid and, of course, stearic acid are inactive, while 9,10 dihydroxystearate is strongly inhibitory (Table 2).

Judging from these few data, it would appear that the geometry of the molecule and the electronegativity of the substituent are among the chief factors that determine biological activity. The exact location of the substituent in the fatty acid chain seems to be of lesser importance, since the 9- and 10-hydroxy derivatives have equal growth-promoting activity, and since the 12-hydroxy compound is also slightly active. The physical properties of the acids do not seem to play a significant role, since the hydroxy acids, for example, melt at very much higher temperatures than oleic acid.

Whether oleic acid and the compounds which can replace it exert their growth promoting effect as free acids or in some combined form, is not known. Cells grown with 1-C^{14} oleate contain radioactivity in every one of the lipid fractions obtainable by silicic acid chromatography. On the other hand, in the experiments with labeled hydroxy acid, the isotopic distribution pattern is very much simpler, radioactivity appearing only in the free acid fraction and in a fraction emerging with the triglycerides. We have not yet elucidated the structure of the esterified form of the hydroxy acid, but have shown that it is not a glyceride or a sterol ester.[2]

The ability of hydroxystearic acids to substitute for oleate in fatty acid-dependent systems has been demonstrated in one other case. As Delmain et al. have shown, a sarcina species which they have isolated will grow

[2] The esterified form of the hydroxy acid has now been identified as ethyl acetoxystearate.

with a markedly reduced lag phase if the synthetic medium is supplemented with a small amount of oleate (16). Saturated fatty acids are inactive but both the 9- and the 10-hydroxystearates are as effective in shortening the lag phase of growth as oleate. Labeled hydroxystearic acid is not converted to oleate by sarcina and therefore, the functional equivalence is not a reflection of metabolic conversion. It is of interest, on the other hand, that in lactobacilli deprived of biotin and requiring an unsaturated fatty acid for growth (17, 18) the hydroxystearates cannot substitute for oleate.

MULTIPLY UNSATURATED FATTY ACIDS

A recent study in our laboratory has been concerned with mechanisms that introduce additional double bonds into aliphatic chains. Investigating, as an example, the biosynthesis of linoleic acid, we assumed that the diene system is established in two steps and that the most probable immediate precursor of linoleate is oleic acid. The organism chosen for studying the conversion of oleate to multiply unsaturated acids was *Torulopsis utilis* because the linoleic acid content of this yeast is normally very high. On incubation of growing or resting torulopsis cells with 1-C^{14} oleate, labeled linoleate was formed in high yield, with some evidence for desaturation proceeding further to the triply unsaturated linoleic acid (19). When resting cells were used, the introduction of the double bond into oleic acid was found to depend on an atmosphere of oxygen, suggesting that the mechanism is oxidative as in the desaturation of stearate to oleate. Thus, even though a double bond is already present in the molecule, the further desaturation cannot occur by an anaerobic dehydrogenation. This absence of an activating effect is not surprising, because in linoleic acid the second double bond is in divinylmethane relationship to the first. On the other hand, it is conceivable that the second double of a conjugated diene system, as in the carotenes, is introduced by a different mechanism.

Attempts to implicate a hydroxy olefinic acid as a precursor of linoleate have so far given negative results. Ricinoleic acid, the naturally occurring 12-hydroxy-Δ^9 decenoic acid, does not appear to be an intermediate in the conversion of oleate to linoleate, although one might have supposed that it arises from oleate by hydroxylation and forms linoleate by dehydration. The failure of this reaction to occur presents the same dilemma as the metabolic inertia of hydroxystearic acids in the synthesis of oleic acid. The possibility that oxygenated products, other than the free hydroxy acids, are the intermediates has already been discussed. It is also conceivable that in both reactions oxygen serves as the immediate electron or hydrogen acceptor without becoming covalently linked to the substrate.

ANAEROBIC MECHANISMS

The oxidative pathway for the biosynthesis of unsaturated fatty acids, as demonstrated for the enzyme system of yeast, presumably exists also in other biological systems which use the long-chain saturated fatty acids as direct presursors for the corresponding olefins, i.e. in animals (4, 20) and in plants.[3] Anaerobic microorganisms, on the other hand, must either accomplish the same type of transformation without oxygen or possess alternative mechanisms for synthesizing unsaturated fatty acids, provided that these compounds are essential constituents of anaerobic as well as of aerobic organisms. The detailed fatty acid composition of bacterial lipids, particularly of anaerobes, is known only in a few instances. The most thorough study is that of Hofmann and his collaborators, who have shown that *cis* vaccenic acid, the Δ^{11} isomer of oleic acid, is the principal unsaturated fatty acid in a number of Lactobacillus species (21) and in *Agrobacter tumefaciens* (22). Lactobacilli also contain a C_{19} Δ^{11} cyclopropane acid (lactobacillic acid) (23) which is produced from vaccenic acid by methylation (24).

TABLE 3. *Position of Double Bonds in Fatty Acids of Microorganisms**

Organism	C_{16} Δ^7	C_{16} Δ^9	C_{18} Δ^9	C_{18} Δ^{11}	Mechanism of Synthesis
Lactobacillus plantarum†		+		+	anaerobic
Escherichia coli		+		+	anaerobic
Pseudomonas fluorescens		+		+	anaerobic
Clostridium butyricum	+	+	+	+	anaerobic
Mycobacterium phlei		?	+		aerobic
Sacharomyces cerevisiae		+	+		aerobic
Anabaena variabilis		+	+		aerobic

* Determined by KIO_4-$KMnO_4$ oxidation of the unsaturated fatty acids and gas chromatographic identification of the resulting dicarboxylic acids. † From ref. (21).

Analyzing the fatty acids of several representative bacterial species by vapor phase chromatography and by oxidative degradation, we have found vaccenic acid to be widely distributed. It is present in the lipids of the obligate anaerobe *C. butyricum*, in *E. coli*, a facultative anaerobe, and in *Pseudomonas fluorescens*, which is an obligate aerobe. In two instances, in *S. fecalis* (22) and in *C. butyricum*, the C_{18} monounsaturated fraction contains the Δ^9 isomer, in addition to vaccenic acid. So far only one exception to this wide distribution of vaccenic acid in bacteria has been noted. In several strains of tubercle bacilli (25) and in *Mycobacterium phlei*, oleic acid is the principal and presumably only octadecenoic acid. Double bond isomers of monounsaturated C_{16} acids have not been previously reported.

However, we have now obtained evidence for the presence of a Δ^7 hexadecenoic acid in the lipids of *C. butyricum* and hence palmitoleic acid is no longer the sole naturally occurring C_{16} monounsaturated acid, though it is certainly the most common one. The biogenetic significance of the existence of double bond isomers both in the C_{16} and the C_{18} series will be discussed

[3] This problem has not yet been investigated extensively in higher plants but we have shown that in the blue-green algae *Anabaena variabilis* oleic acid is formed by desaturation of stearate.

below. Some of the relevant information is given in Table 3.

In microorganisms which normally contain vaccenic acid, e.g. *C. butyricum*, *L. plantarum* or *E. coli*, the environmental atmosphere has no noticeable influence on the relative proportions of saturated and unsaturated acids in the cellular lipids (Table 4), or on the rate at which they are synthesized from acetate. This is in striking contrast to the strict oxygen-dependence of the processes by which palmitoleate and oleate are formed in yeast. For the synthesis of palmitoleic acid, two separate pathways seem to exist; the aerobic desaturation of palmitate which is employed by yeast, and probably also by plants and animals, and an anaerobic mechanism employed by certain bacteria. As to the C_{18} monounsaturated acids, the anaerobic bacterial pathway is apparently capable of producing both the Δ^9 and the Δ^{11} isomers, whereas the aerobic process invariably yields the Δ^9 acid. It appears to be a typical feature of the aerobic process that it introduces the double bond at the 9th and 10th carbon atom from the carboxyl group whether the saturated precursor has 16 or 18 carbon atoms.

Among the mechanisms which bacteria might employ for the synthesis of olefinic acids, we first considered the possibility of a direct hydrogen elimination, in analogy to the reactions catalyzed by succinic or acyl CoA dehydrogenase. It appears, however, that flavoprotein-linked dehydrogenations operate only when the double bond is introduced α,β to a carbonyl group. Labeled palmitate or stearate, added to the growth medium of *L. plantarum*, *E. coli*, or *C. butyricum*, are not significantly

TABLE 4. *Fatty Acids of Anaerobically Grown Microorganisms**

	C. butyricum	*E. coli*	*L. plantarum*
Saturated			
C_{16}	49.0	40	39.1
C_{18}	6.2	1	2.3
Unsaturated			
C'_{16}	17.0	3	4.5
C'_{18}	7.9	3	29.2
Cyclopropane acids†			
C_{17}	9.0	20	
C_{19}	5.2	27	24.9

* The fatty acid compositions of aerobically grown *E. coli*, and *L. planatarum* do not differ significantly from those shown above. † In lactobacilli (24) and *E. coli*, these acids are formed by methylation of olefinic acids and they are, therefore, "unsaturated."

TABLE 5. *Lack of Desaturation of Stearate in Anaerobic Bacteria*

	*L. plantarum** c.p.m. × 10^{-3}	*C. butyricum* c.p.m. × 10^{-3}
C^{14} stearate added	1700	2000
C^{14} incorporated	720	500
Total saturated acids	700	490
Total unsaturated acids	<20	20

* The results were the same whether the organisms were grown in air or in strict anaerobiosis.

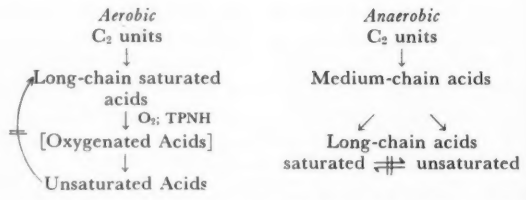

FIG. 1. Mechanisms of synthesis of long-chain fatty acids in microorganisms.

transformed to unsaturated acids, either under aerobic or anaerobic conditions (Table 5). This lack of conversion cannot be attributed to permeability factors, since the saturated acids are efficiently incorporated into the cellular lipids of these organisms. We have also failed to obtain any evidence for the dehydrogenation of saturated fatty acids or of their CoA esters in cell-free extracts of *C. kluyveri*. Thus there is little doubt that the mechanism which produces unsaturated fatty acids under anaerobic conditions does not use the corresponding saturated fatty acids as precursors. The remaining alternative is a separate or independent process of condensation, which affords the olefinic acids directly rather than by way of the saturated acids.

Following a suggestion by Hofmann et al. (26), we can visualize a modified process of chain-building which uses a short or medium-length unsaturated acyl CoA intermediate, condensing it directly with additional malonyl CoA units. The double bond is not reduced as it is in the synthesis of the saturated acids, but is retained and gradually moved from the carboxyl end as more C_2 units are being added (Fig. 1). To account more specifically for the position of the double bonds in the C_{16} and C_{18} fatty acids found in bacterial sources, it is necessary to invoke a crotonase-like enzyme capable of dehydrating a C_{10} or C_{12} hydroxyacyl CoA intermediate to the β,γ rather than to the α,β olefin. Starting from the C_{10} unsaturated intermediate, we can, thus, rationalize the formation of palmitoleic acid and vaccenic acid by addition of 3 and 4 C_2 units, respectively (Fig. 2) and elongating a C_{12} unsaturated acid, can account for the Δ^7-hexadecenoic acid of *C. butyricum* and for oleic acid by attachment of 2 and 3 C_2 units, respectively (Fig. 3).

It may be relevant to the above mechanism, that β-hydroxydecenoic acid occurs commonly in bacteria and, moreover, that it belongs to the D-series (27, 28), which is opposite to the configuration of the β-hydroxy acids arising in fatty acid oxidation. Recently we have obtained evidence also for the occurence of β-hydroxylauric acid in a Pseudomonas species. It will be of interest to see whether bacterial crotonase acts on these D-hydroxy acids and to ascertain whether the α,β-, the β,γ- or both unsaturated acids are the products.[4]

[4] According to evidence presented at this symposium by F. Lynen, D-β-hydroxy acids are intermediates in the synthesis of saturated fatty acids in yeast. It, therefore, seems likely that in various bacterial systems β-D-hydroxy acids are the common precursors of both saturated and unsaturated fatty acids.

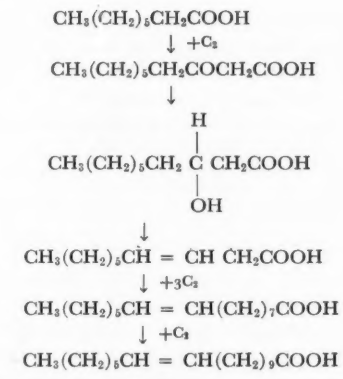

FIG. 2. Anaerobic mechanism for the synthesis of olefinic acids from octanoic acid.

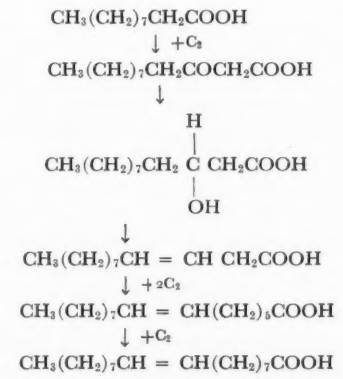

FIG. 3. Anaerobic mechanism for the synthesis of olefinic acids from decanoic acid.

The mechanism presented here is consistent with a number of observations made both in Hofmann's laboratory and in our own. As Hofmann et al. have shown, several shorter-chain unsaturated acids, all having the double bonds at the same distance from the methyl terminal of the molecule, can replace vaccenic acid or oleic acid as growth factors for lactobacilli (26). This fits the proposed scheme, although the data do not distinguish between chain elongation and direct utilization of the unchanged acids. We have found that in lactobacilli, palmitoleic acid undergoes chain elongation to an unsaturated fatty acid, which is apparently vaccenic acid. This is in line with the prediction that in the anaerobic path a given olefinic acid arises by C_2 addition to the carboxyl end of the lower homologue, leaving the double bond at the same distance from the methyl end of the molecule. The general concept of a separate synthetic pathway to unsaturated fatty acids in certain bacteria is further and more strongly supported by observations made with homologous saturated fatty acids in *C. butyricum* (29). When these bacteria are grown with 1-C^{14} octanoate or 1-C^{14} decanoate, both the saturated and the unsaturated long chain fatty acids become labeled and, as degradation data show, they contain the bulk of the C^{14} within the fatty acid chain. The C_8 and the C_{10} fatty acids are, therefore, elongated to both saturated and unsaturated fatty acids. The C_{12} and C_{14} saturated acids are also converted to longer chains, but in these cases only the saturated acids are labeled (Table 6). This strongly suggests that the pathways to saturated and unsaturated acids separate at the C_8 and the C_{10} level, and also that the olefinic acids cannot be formed by dehydrogenation of their saturated counterparts.

As already mentioned, the unsaturated fatty acids of *C. butyricum* consist of two pairs of double bond isomers. The schemes shown in Figs. 2 and 3 stipulate that octanoic acid is the precursor of Δ^9-hexadecenoic and of Δ^{11}-octadecenoic acid, while decanoic acid is the precursor of Δ^7-hexadecenoic and of Δ^9-octadecenoic acid. The validity of these pathways has been tested as follows: From the unsaturated fatty acids of *C. butyricum* grown in the presence of 1-C^{14} decanoate, the C'_{16} and the C'_{18} fractions were obtained by vapor phase chromatography and then degraded separately by the KIO_4-$KMnO_4$ procedure. The C'_{16} and C'_{18} fractions each yield two dicarboxylic acids which are separable by vapor phase chromatography, and can then be assayed for C^{14}. The C_7 dicarboxylic acid from the C'_{16} fraction and the C_9 dicarboxylic acid from the C'_{18} fraction were radioactive, whereas the C_9 and C_{11} dicarboxylic acids derived, respectively, from the C'_{16} and C'_{18} fractions were unlabeled. These findings are in accord with prediction (Fig. 3), and provide the strongest support so far for the proposed chain-elongation of β,γ unsaturated acids. They show that the position of the double bond in these bacterial fatty acids is determined by the initial chain-length of the saturated fatty acid precursor (C_8 or C_{10}), and also that the occurrence of double bond isomers is not due to double bond shifts.

The mechanism proposed here relies heavily on the position of the double bonds in the fatty acids isolated from a rather small number of microorganisms. Biosynthetic arguments based on the structure of metabolic end products are notoriously hazardous, but in the present case they can be made with some confidence because secondary double bond shifts do not seem to occur. The possibility that some of the monounsaturated

TABLE 6. *Incorporation of Homologous Saturated Acids into Long-chain (C_{16} + C_{18}) Acids of C. butyricum*

C^{14} Acid Added to Growth Medium*	% of Incorporated C^{14} in Saturated Acids	% of Incorporated C^{14} in Unsaturated Acids†
C_2	60	40
C_8	75	25
C_{10}	80	20
C_{12}	96	4
C_{14}	96	4
C_{16}	97	3
C_{18}	99	1

* All the acids were labeled in the carboxyl group. † Plus cyclopropane acids.

acids represent reduction products of more highly unsaturated acids is also unlikely, since we have failed to detect any multiply unsaturated acids in the lipids of bacteria.

It deserves special comment that the acquisition of an aerobic metabolism does not necessarily involve a shift from the anaerobic to the aerobic mechanism for synthesizing unsaturated fatty acids. *E. coli* and *Pseudomonas fluorescens*, although they utilize oxygen as terminal electron acceptor for energy production, have retained the anaerobic mode of synthesis. On the other hand, the anaerobic route to unsaturated fatty acids is not common to all bacterial species. In mycobacteria, the pathway to unsaturated acids is oxidative and presumably the same as in yeast, plants, and animal tissues. Further comparative studies are necessary to ascertain whether it is generally true that the anaerobic route is characteristic for the Eubacteriales and the oxidative path for Actinomycetales and all higher forms.

The postulated existence of independent mechanisms for the synthesis of saturated and unsaturated fatty acids has some bearing on the well-known relationship between biotin and oleic acid in the metabolism of certain bacteria. In *Lactobacilli* and in *C. butyricum* (17, 18), the biotin requirement can be met under certain conditions by vaccenic or oleic acid, but not by saturated fatty acids. There is no reason to believe that biotin plays a role in the reactions specifically concerned with the introduction of double bonds. As experiments with labeled acetate have shown, lactobacilli cannot synthesize fatty acids, either saturated or unsaturated, in the absence of added biotin, but cells grown with an oleic acid supplement contain, nonetheless, saturated fatty acids in large amounts. The possibility that they are derived from oleic acid by reduction has been ruled out by appropriate experiments with labeled oleate and palmitoleate. The major metabolic route of unsaturated acids in these organisms is the methylation to cyclopropane acids (Table 7). Resistance of double bonds to enzymatic reduction, when they are in an isolated position in the fatty acid chain, has previously been noted with oleic acid in animal tissues (30), in yeast (19), and with vaccenic acid in *L. arabinosus* (31), and, thus, appears to be a general phenomenon. Conceivably, the centers of unsaturation at carbon atoms 9 and 10 and at 11 and 12 are too far removed from the activating carboxyl group to permit polarization of the double bond, which, in turn, may be the prerequisite for an attack by hydride ion.

Since the lactobacilli are unable to obtain saturated

TABLE 7. *Transformations of 1-C^{14} Oleic Acid*

	L. plantarum c.p.m. × 10^{-3}	*M. phlei* c.p.m. × 10^{-6}
C^{14} oleate added	760	1350
Total fatty acids	220	338
Palmitic a.	1	20
Stearic a.	2	2
C_{19} cyclopropane a.	75	
10-Methylstearic a.		200

1) β-oxidation (animal tissues and plants)

2) $CH_3(CH_2)_nCH{=}CH(CH_2)_nCOOH \xrightarrow{H_2} CH_3(CH_2)_nCH_2CH_2(CH_2)_nCOOH$

3) $CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH \rightarrow$ dienoic and polyenoic acids (yeast and higher plants)

4) $CH_3(CH_2)_nCH{=}CH(CH_2)_nCOOH \rightarrow$
C_{19} branched chain acids (bacteria; plants?)
a) cyclopropane acids (*Eubacteriales*)
b) methylstearic acid (Mycobacteria)

FIG. 4. Reactions of unsaturated fatty acids.

fatty acids by reducing olefinic acids or by total synthesis because of the lack of biotin, the saturated acids found in the cells under these special conditions must have an external origin. The most probable sources are the Tweens, which appear to be essential and not replaceable by other types of detergents if lactobacilli are to grow on biotin-less, oleic acid-supplemented media.[5] A similar nutritional relationship between biotin and oleic acid exists in *C. butyricum* (18), but in this organism biotin synthesis is apparently not blocked completely so that saturated acids can still be formed (29). At any rate, these organisms are clearly unable to interconvert long-chain saturated and unsaturated fatty acids and hence employ for their synthesis separate pathways. This particular point has also been established very clearly by Shorb and Lund for 2 strains of Trichomonas. When cultured on biotin-free media these protozoa require both a saturated and unsaturated fatty acid for growth (32).

As far as the further metabolism of long-chain unsaturated fatty acids is concerned, the evidence suggests that it can take strikingly different directions in various biological systems (Fig. 4). In animal tissues, oxidative breakdown and complete combustion is the main route (33). On a very minor scale, there is a conversion to dienoic acids, which are isomeric with linoleate (34). In *Torulopsis utilis* and in blue-green algae we have observed a very rapid conversion of oleate to linoleate and linolenate but scarcely any breakdown to smaller units. Bacteria also have a limited ability to oxidize long-chain olefinic acids, although this subject has not been studied sufficiently to allow any generalization (35). In several bacterial species, the most conspicuous transformation of olefinic acids is the addition of methyl groups to the double bond, yielding cyclopropane acids in some cases [Lactobacilli (24) and *E. coli*] and 10-methylstearic acid in others [mycobacteria (36)]. As has already been pointed out, a reduction of isolated double bonds has not been observed in any of the systems so far investigated.

A final point of interest is that dienoic or more highly unsaturated fatty acids have not been found in bacteria which use the anaerobic route for synthesizing olefinic

[5] The part played by the Tweens appears to be more complex than to serve merely as a source of saturated fatty acids. Lactobacilli fail to grow on biotin-less media supplemented with unsaturated fatty acids, and with a saturated fatty acid in place of Tween.

acids. Apparently multiply unsaturated acids can be formed only by oxidative processes of the type demonstrated for the conversion of oleic acid to linoleic acid. These mechanisms seem to exist only in the more highly differentiated aerobic forms of life.

The principal findings made in the course of these studies on the synthesis of unsaturated fatty acids in various biological systems may be summarized as follows:

1) Two separate pathways exist for the synthesis of monounsaturated fatty acids. One of these produces oleic and palmitoleic acids by oxidative desaturation of stearate and palmitate, respectively. It is the only or predominant route in yeast, blue-green algae, and in some aerobic bacteria (Mycobacteria).

2) Anaerobes, facultative aerobes, and some obligate aerobic bacteria lack the ability to desaturate palmitate or stearate, either in the absence or presence of oxygen. In these organisms, olefinic acids are synthesized under strictly anaerobic conditions by a route that branches from or is entirely independent of the pathway that produces the long-chain saturated fatty acids.

3) Multiply unsaturated fatty acids are formed from oleic acid by an oxidative mechanism. They are not synthesized or found in organisms in which vaccenic acid is the principal C_{18} unsaturated fatty acid, nor is there evidence that they occur in any bacterial lipids.

We are indebted to Professor K. Hofmann for samples of lactobacillic acid and vaccenic acid; to Professor S. Hünig for a sample of D,L-10-methylstearic acid, and to Doctors L. W. Beck and F. W. Mattson for a sample of uniformly-labeled palmitoleic acid.

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Chylomicron metabolism[1]

T. OLIVECRONA, E. P. GEORGE[2] AND B. BORGSTRÖM
Department of Physiological Chemistry, University of Lund, Lund, Sweden

AFTER ABSORPTION FROM THE SMALL INTESTINE, the long chain fatty acids of the dietary fat are given off to the lymphatics, draining the small intestine in the form of small light scattering particles known as the chylomicrons. Around 90% of the chylomicrons are made up by triglycerides, the remainder being phospholipids, cholesterol, cholesterol esters, and a small amount of protein. The chylomicrons constitute a family of particles of low density which can be collected by centrifugation. By definition, the chylomicrons belong to the low density lipoproteins with Sf values >400.

The chylomicrons enter the systemic circulation by way of the jugular vein and are rapidly metabolized. The rate of metabolism seems to vary with the species. In the human, the rate of entry after a lipid rich meal usually exceeds the rate of removal and the blood becomes turbid. In the rat, the rate of removal is usually equal to the rate of entry and a chylomicronemia is seldom seen.

The metabolism of chylomicrons has attracted much attention during recent years. The removal from the circulation of fatty acid labeled chylomicrons was studied in 1956 by Havel and Fredrickson (1) in the dog, and French and Morris (2) in the rat. The first part of the disappearance curves followed a single exponential function, with a half-life between 4–14 min in the rat and 15–24 min in the dog; the half-life being inversely proportional to the amount injected. In French and Morris' work, the later course of the disappearance curve was complex and about 5% of the activity was removed at a much slower rate.

What does the slower part of the curve represent? Two possibilities are apparent to explain this type of curve: either an inhomogenity in the injected material with different disappearance rates, or a recirculation of active material. The slow component could be the phospholipid fraction, which, in the case of palmitic acid labeled chylomicrons, represents around 4% of the activity of the chyle. This would be in agreement with the finding of McCandless and Zilversmit (3), who reported a slow disappearance rate of the phospholipids of chyle (labeled by P^{32}); but in contrast to the statement by Havel and Fredrickson of 1956 (1) that the triglyceride and phospholipid moieties of the chylomicrons disappeared from the plasma at the same rate. In the work of the latter authors, in spite of a rapid turnover of the chylomicrons, little radioactivity appeared in the other plasma lipoprotein fractions, indicating that recirculation is of little importance in the dog. In the work of French and Morris (2), on the other hand, only total lipid radioactivity was measured. If the phospholipid fatty acids of the chylomicron were chiefly responsible for the slow phase of radioactivity disappearance, the break of the curve should be different with different labeled fatty acids. This reasoning is based on the fact that different dietary fatty acids are incorporated to different degree into the chylomicron phospholipids. Thus, it has been found that 4% of the total chylomicron activity is present in phospholipids when C^{14}-palmitic acid is present in the dietary fat, compared to 20% for labeled stearic acid (4).

In the experiment shown in Fig. 1, rats were injected with lymph obtained from rats fed glycerides containing trace amounts of C^{14}-palmitic acid and T-stearic acid. Plasma lipids recovered at different times after the injection were separated into glycerides, phospholipids, and free fatty acids (5), and their radioactivity was determined. It is apparent that the break in the disappearance curves is very different for the palmitic and stearic acid label, and that it is the magnitude of incorporation of label into the phospholipids which is the major determining factor. The disappearance curve for the glyceride fraction follows a single exponential until a very low level and, therefore, inhomogenity or recirculation in this fraction is of a low magnitude. A certain recirculation of the injected acids is seen in the free fatty acid fraction, as has earlier been reported (1, 6). The extent of recirculation in both the free fatty acid and glyceride fraction is, however, different for palmitic and stearic acid, the latter being recirculated to a much lesser extent. This is apparent in the curve to the right in Fig. 1, showing the ratio of palmitic to stearic acid activity. This ratio is constant during the first 30 min that is the time of chylomicron removal.

The mechanism of the chylomicron clearing has been

[1] The work on which this article is based has been supported by grants from the Swedish Medical Research Council and the U. S. Public Health Service Met-5302).

[2] Visiting Scientist. Permanent address: Dept. of Physics, St. Vincents Hospital, Sydney, Australia.

The skillful technical assistance of Irene Lindell, Kerstin Rosendahl, and Agneta Celander is gratefully acknowledged. Dr. George expresses his thanks to the postgraduate committee in Medicine of the University of Sydney for a grant toward the cost of travel.

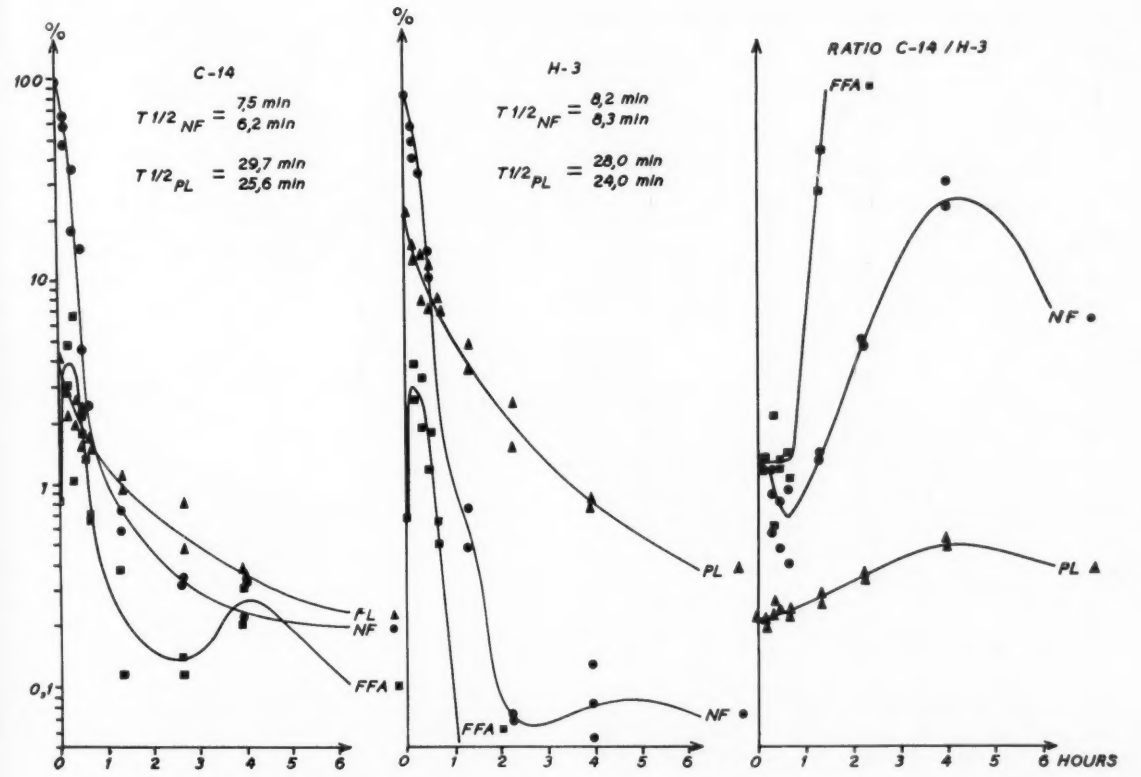

FIG. 1. Activity-time relationship of plasma neutral fat (NF), free fatty acids (FFA), and phospholipids (PL), after injecting rats with 1-C^{14}-palmitic-, H-3-stearic acid labeled chylomicrons

much discussed against the background of the finding of Hahn in 1943 (7) of a heparin induced clearing factor. This enzyme, which has also been found present in blood of fasting animals and man, and in increased amount during fat absorption, has been classified as a lipoprotein lipase and splits the ester bonds of chylomicron glycerides with specificity for the ester bonds with primary hydroxyls (8). Different findings have been taken to indicate the importance of the clearing factor for the chylomicron removal. The most important among these are the appearance of radioactivity in the free fatty acid fraction of blood during the removal of labeled chylomicrons (1, 6), the appearance of mono- and diglycerides (9–11) in the plasma lipides during alimentary lipemia, the increased rate of disappearance of chylomicron fat after the injection of heparin, and the decreased rate of disappearance after protemine injection (12).

The quantitative evaluation of the importance of the clearing factor has, however, been rather difficult. From the amount of clearing factor present in rat blood during fat absorption, it has been calculated that up to 30% of the chylomicron triglyceride could be hydrolyzed to unesterified fatty acids in the blood (13, 14). Engelberg (15) has made similar calculations and arrived at the conclusion that the intravascular clearing factor may account for the splitting of 4 to 5 g of neutral fat per hr in the human. This transformation of in vitro data to in vivo is, of course, very difficult to evaluate.

As has been pointed out by Engelberg (15), the occurrence of mono- and diglycerides in blood during chylomicron disappearance (9–11) is not conclusive evidence for intravascular lipolysis, as the partial glycerides may have been original constituents of the chylomicrons. Such a mechanism is substantiated by the results shown in Fig. 2. Rat chylomicrons, labeled both in the glycerol (C^{14}) and fatty acid part (T-palmitic acid), were injected into rats and the plasma lipids recovered after different time intervals. The glycerides of the plasma lipids were separated on thin layer silicic acid chromatography and the tri-, di- and monoglyceride spots scraped off, eluted, and their activity counted. The results (Fig. 2) indicate that the diglyceride initially present in the chylomicrons is removed at a constant rate and that very little, if any, diglyceride is formed during the clearing of the chylomicrons from the circulation. The initial increase in the diglyceride-C^{14} curve is probably not significant. The shape of the tritium-fatty acid-monoglyceride curve might indicate the formation of some monoglycerides during the same conditions. The C^{14}-glycerol-monoglyceride activity was too low to give significant counts.

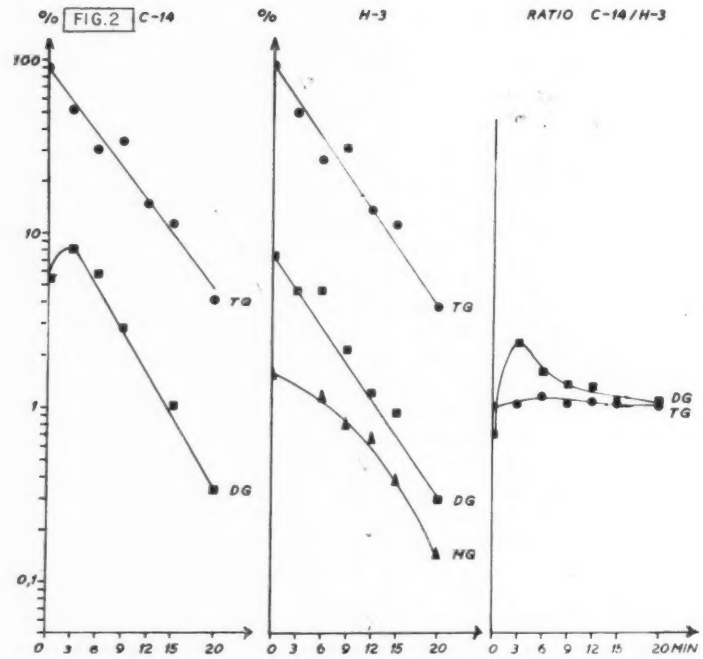

FIG. 2. Activity-time relationship for plasma tri-, di- and monoglycerides after injecting rats C-14-glycerol-H-3-palmitic acid labeled chylomicrons. The glycerides were separated by thin-layer silicic acid chromatography, and the silicic acid scraped off and counted.

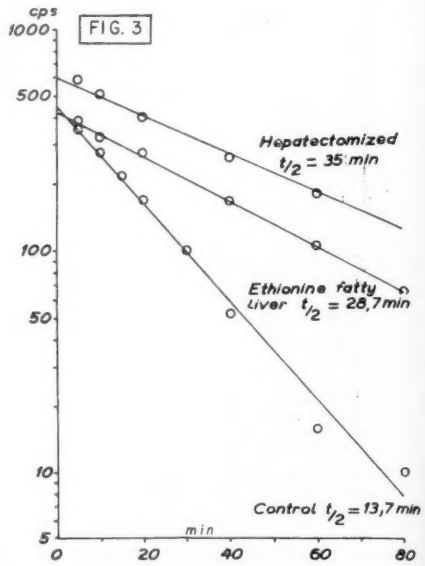

FIG. 3. Disappearance curve for 1-C-14-palmitic acid labeled chylomicrons injected into normal rats, hepatectomized rats and rats with ethionine induced fatty livers. Each curve calculated from experiments on two animals (21).

As earlier mentioned, Havel and Fredrickson (1956) (1) found that during clearing from the blood in dogs of fatty acid labeled chylomicrons the specific activity of the blood free fatty acid fraction increased to figures exceeding 50% of the specific activity of the injected glyceride fatty acid. This finding, in combination with the rapid turnover of the free fatty acid fraction indicated to these authors an intimate relationship between the removal of chylomicrons and the hydrolysis of their glycerides, and that, "an appreciable portion of the cleared chylomicron triglyceride may be re-transported through the plasma as unesterified fatty acid during the clearing process." The authors did not believe that the hydrolysis of the chylomicrons occurred in the circulating blood, as they were unable to observe evidence of a continued lipolysis in vitro. In view of the results of Engelberg (16) on clearing factor inactivation, this argument does not seem quite conclusive.

In a later study, however, Fredrickson et al. (6) obtained results which were calculated to mean that retransport as free fatty acids is not obligatory in the utilization of exogeneous triglyceride. From the data obtained, it was calculated that 50% of injected chylomicron triglyceride appeared in the blood as free fatty acids. These figures are based on experiments in which the specific activity curves for blood free fatty acids and expired CO_2 were compared when the labeled acid was injected as free fatty acid or chylomicron. It was found that the ratio of flux of label between plasma free fatty acid and expired CO_2 during a 4–6 hr period was much less when chylomicrons were injected. It was also found that the specific activity of the blood free fatty acid fraction, after injecting labeled chylomicrons, dropped below that of the expired CO_2. Studies of the tissue distribution of injected labeled free palmitic acid and palmitic acid labeled chylomicrons also led Bragdon and Gordon (17) to the conclusion that the bulk of the chylomicrons do not undergo intravascular hydrolysis, but are removed intact from the circulation.

The interpretation of the above calculations has recently been criticized by Engelberg (15), who concluded that available data support the thesis that the major normal pathway of chylomicron removal is by way of intravascular enzymatic lipolysis by clearing factor.

The finding that chylomicron glycerides are taken up intact by the liver, as evidenced by experiments with glycerol-fatty acid labeled chylomicrons (18), however, definitely proves that chylomicron glycerides, partly at least, are removed intact from the circulation. The percentage of activity in the liver in these experiments reached a maximum of around 45 20 min after injection. The rapid decline of the activity in the liver glycerides means by necessity that more than 45% of the injected chylomicron activity must have passed the liver. A similar pathway of chylomicron removal is also supported

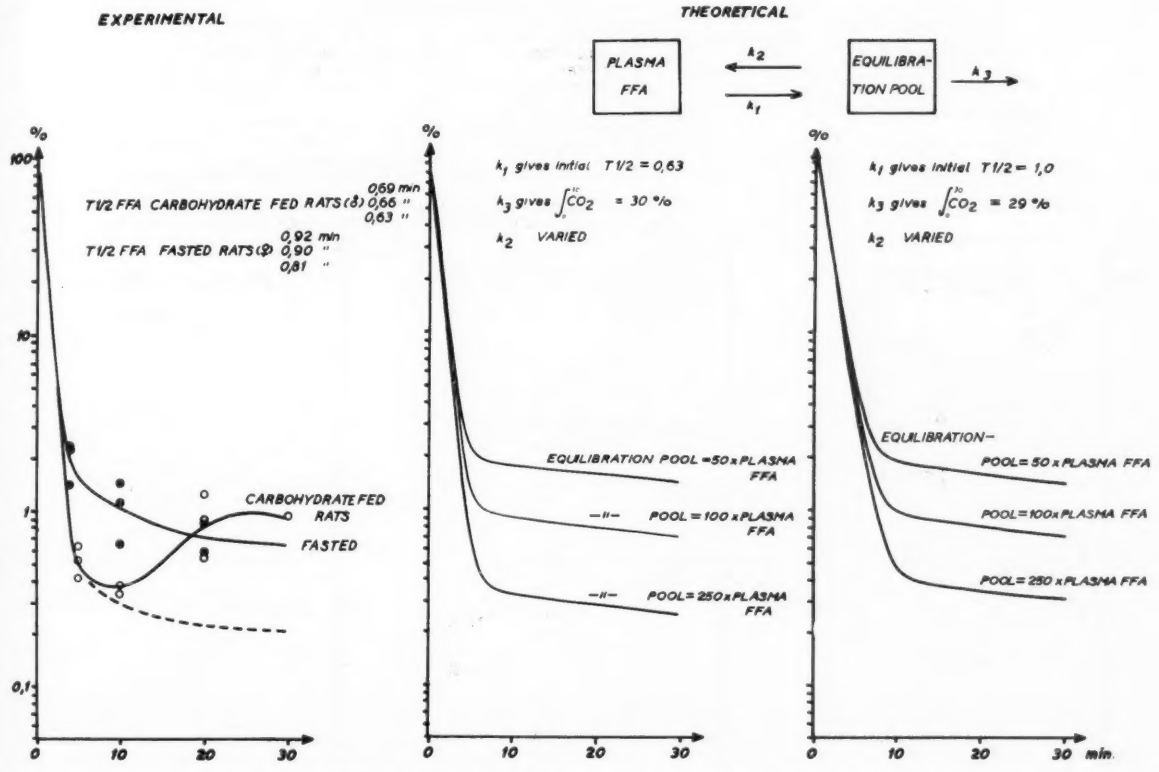

FIG. 4. Disappearance curves for injected albumin bound free 1-C^{14}-palmitic acid in rats. Left experimental curves, middle and right curves, calculated according to the data shown.

by the data in Fig. 1 and the results reported at this meeting by Dustin and Fredrickson (19) that when chylomicrons labeled with two different fatty acids are injected, the liver glycerides initially contain these acids in the same ratio as in the chylomicrons.

The direct importance of the liver in chylomicron removal is also stressed by the decreased rate of removal of chylomicrons in hepatectomized rats and rats with ethionine induced fatty livers (20) (Fig. 3).

To get an opinion of the quantitative importance of the different pathways of chylomicron removal, we have determined the plasma glyceride and free fatty acid activity curves of rats injected with fatty acid labeled chylomicrons. We have then made up some different models for chylomicron removal and, with the aid of a digital computer, obtained the corresponding activity curves. The rate constant for removal of chylomicron fatty acids entering the plasma free fatty acid pool has been obtained experimentally, after injecting rats with albumin bound C^{14}-palmitic acid. Figure 4 shows the experimental curves for free fatty acid disappearance, the model used, the curves for different sizes of the equilibration pool, and two different T ½ for the injected free fatty acid corresponding approximately to the disappearance rate in fasted and carbohydrate fed rats.

It is clear from this experiment that the equilibration pool of plasma free fatty acids is between 100 and 250 times the plasma free fatty acid pool. The figure 250 has then been used in the calculation in Fig. 5. The left part of this figure gives the experimental data for glyceride and free fatty acid activity, after injecting rats with fatty acid labeled chylomicrons. The open and closed circles correspond to experiments with different amounts of the same chylomicron suspension. The calculations only refer to the experiment with the open circles, in which approximately 60 mg of chylomicron fatty acids were injected over 1 min. The two calculated sets of curves in Fig. 5 refer to the model shown with different percentages of intravascular chylomicron hydrolysis, from 10 to 100%. The difference in the two sets of curves is the T ½ for the free fatty acid fraction. If these two sets of curves for plasma free fatty acid activity are compared with the one experimentally found (left in the figure), it is apparent that intravascular lipolysis must be of a minor order, as the theoretically obtained maximum for the free fatty acid activity is much too early. This means that the chylomicron hydrolysis must be delayed leading to a later appearance of labeled free fatty acids in the circulation.

Figure 6 contains two different models of chylomicron metabolism. The curve in the middle represents a 100% uptake of intact chylomicrons, which then are hydro-

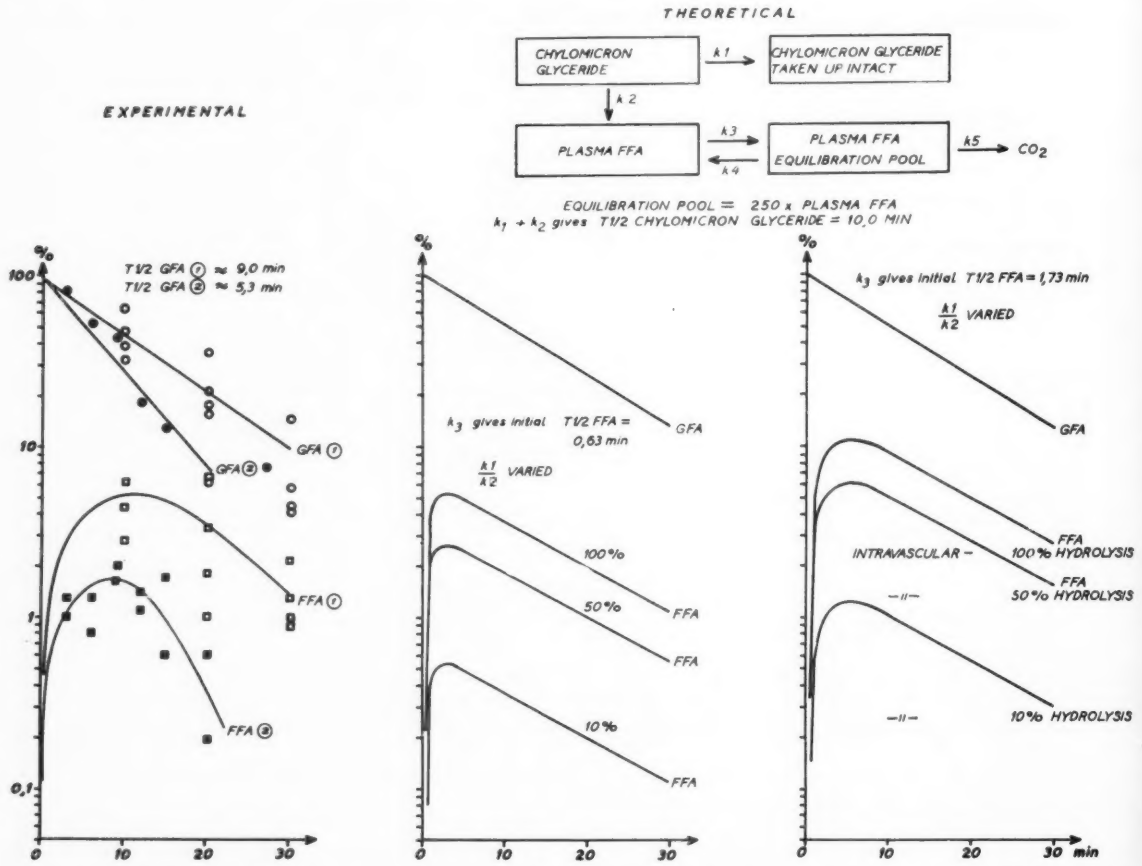

FIG. 5. Activity-time relationship for plasma glyceride fatty acids and free fatty acids after injecting 1-C^{14}-palmitic acid labeled chylomicrons to carbohydrate fed rats (left). The two curves ● and ○ refer to the injection of the same chylomicron suspension in amounts related as 1 to 2. The curves in the middle and to the right have been calculated according to the model and the data given representing different extent of intravascular hydrolysis.

lyzed and mixed in the plasma free fatty acid equilibration pool. The plasma free fatty acid activity curves thus obtained have very little resemblance with the experimental ones, the delay of appearance of labeled free fatty acids being too long. The right part of the same figure demonstrates a model in which the chylomicrons are taken up intact into a pool X, from which their fatty acids are given off directly to plasma without first being mixed with any inert fatty acid or glyceride pool. With a half-life of the pool X of 5 min, the best fit is obtained. Even though this fit is not perfect, it must be realized that we probably do not have one pathway but a combination of different pathways of chylomicron metabolism occurring. It seems probable that the largest part of the chylomicrons are taken up intact from the circulation by the compartment X, from which a large fraction of their fatty acids is rapidly recirculated back to the blood as free fatty acids.

At the site of uptake in the liver, the chylomicron fatty acids are not appreciably diluted by unlabeled acids. This site, therefore, must be rather specific and might be the reticuloendothelial system. Another fraction of the chylomicrons might be taken up by carcass and adipose tissue, where it mixes with a large pool of endogeneous fatty acids, before being recirculated as plasma free fatty acid. Intravascular lipolysis is relatively unimportant. Any exact figures for the magnitude of these different pathways are not at hand at the moment. They may also vary with the chylomicron load, but it seems clear that their order of decreasing magnitude is liver, carcas + adipose tissue, intravascular lipolysis.

With this model, the effect of heparin and protamine on chylomicron removal remains unexplained. It seems, however, possible, as has been pointed out elsewhere, that the importance of intravascular lipolysis, although from a quantitative point of view unimportant, is to change the surface properties of the chylomicrons so that their removal from the circulation is favored (21).

SUMMARY

The pathways of chylomicron disappearance from the blood are discussed.

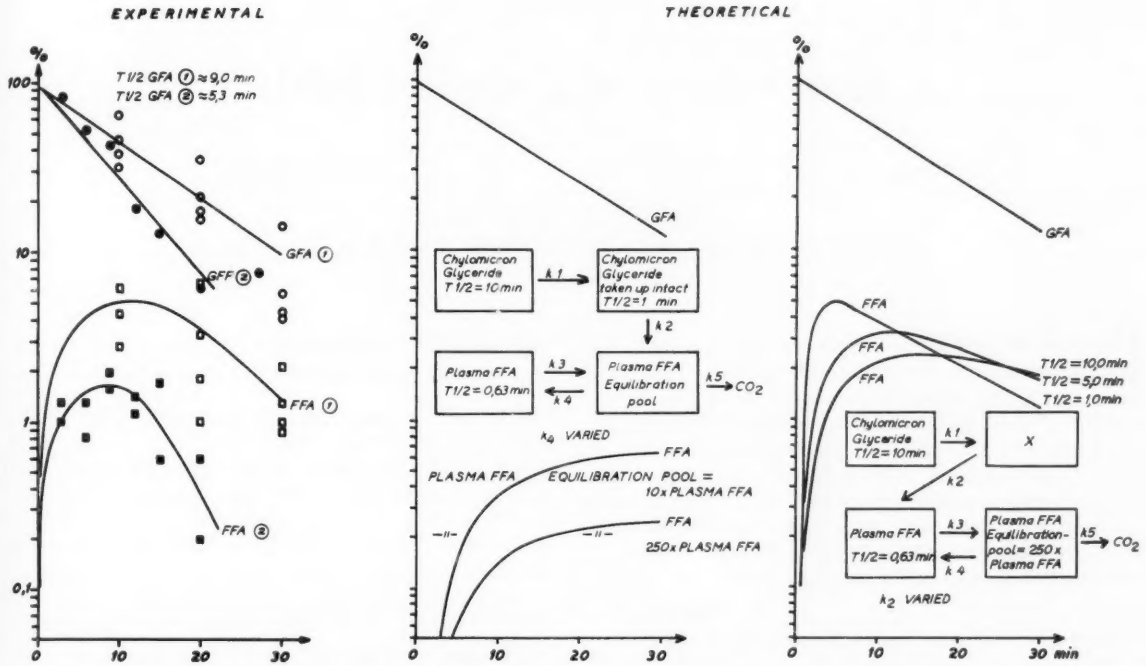

FIG. 6. Experimental data are the same as in Fig. 5. The models, however, are different. Middle curve: chylomicrons taken up intact and equilibrated with the plasma free fatty acid equilibration pool before being returned to the blood as free fatty acids; Right curve: chylomicrons taken up intact in a special pool X from which their fatty acids are given off to the blood (without dilution) with different T/2.

The slowly disappearing component, after injecting fatty acid labeled chylomicrons into rats, has been shown to be caused by the slow disappearance of the chylomicron phospholipid fraction (and to a very minor extent to recirculation of removed fatty acids). The extent of recirculation of chylomicron fatty acids as free fatty acids and glyceride fatty acids is different for palmitic and stearic acid, the latter being recirculated to a much smaller extent.

Our present status of knowledge of chylomicron removal indicates that: *1*) intravascular lipolysis from a quantitative point of view is unimportant; *2*) the largest fraction of the chylomicron glycerides is taken up intact at a special site in the liver, is rapidly hydrolyzed, and their fatty acids given off to the circulation as free fatty acids.

The extra-hepatical tissues may also take up intact chylomicrons. The quantitative relationship between the two pathways is not well established at the present time and may also be dependent on the chylomicron load.

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Biosynthesis of complex lipids

EUGENE P. KENNEDY

Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts

BIOSYNTHESIS OF COMPLEX LIPIDS was last reviewed in a symposium at these Federation Meetings in 1957 (1). Since that time progress in this field has been rapid, as a result of the efforts of many laboratories throughout the world. The present review will be limited to developments since 1957 in three principal fields—the biosynthesis of glycerophosphatides, triglycerides, and sphingolipids. Even with these restrictions, it has not been possible to deal with all the pertinent literature within reasonable limits of time and space.

BIOSYNTHESIS OF GLYCEROPHOSPHATIDES

Presently known pathways for the biosynthesis of glycerophosphatides are shown in Fig. 1. Cytidine-containing coenzymes play a general role in these reaction patterns. So far as is known at present, the de novo generation of the monophosphate diester linkage, an important structural feature of phospholipids, involves a cytidine-containing coenzyme in every instance. The general mechanism for the formation of these bonds is shown in Fig. 2. A requirement for cytidine coenzymes has now been unequivocally demonstrated in the biosynthesis of five major phospholipids, including phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, phosphatidylglycerol, and sphingomyelin. In addition, there is some evidence to suggest that the cytidine coenzymes may be involved in the synthesis of plasmalogenic phospholipids (2).

Two enzyme systems have recently been found to catalyze the conversion of one phospholipid to another. One of these catalyzes the methylation of phosphatidylethanolamine to lecithin (3), and the other the formation of phosphatidylserine from phosphatidylethanolamine and free L-serine (4). However, in both these cases the phosphorus moiety of the newly formed phospholipid is ultimately derived from a cytidine nucleotide—cytidine diphosphate ethanolamine. In neither of these transformations is there a net de novo synthesis of phospholipid.

Many of the enzyme systems utilizing cytidine diphosphate, choline, and cytidine diphosphate ethanolamine have been reviewed elsewhere (1, 5–7) and will not be further considered here.

THE FUNCTION OF CYTIDINE DIPHOSPHATE DIGLYCERIDE IN THE BIOSYNTHESIS OF PHOSPHATIDYLINOSITOL

The first evidence for the enzymatic formation of cytidine diphosphate diglyceride was obtained by Agranoff, Bradley and Brady (8), who found that tritium-labeled CMP could be converted to a chloroform-soluble product in the presence of a particulate enzyme from kidney. However, the amounts of product were too small for adequate characterization, nor was it possible to obtain direct evidence as to the mode of formation or the function of the lipid-soluble cytidine nucleotide in this system.

In an investigation of the biosynthesis of phosphatidylinositol in liver enzyme preparations, Paulus and Kennedy (9) were able to show that L-α-glycerophosphate-P^{32} was converted to phosphatidylinositol by a pathway specifically requiring CTP as a cofactor. It was clear from these results that a pattern of reactions different from those leading to the formation of lecithin and phosphatidylethanolamine must take place in the formation of phosphatidylinositol. In the biosynthesis of the latter phospholipid, the phosphate moiety of L-α-glycerophosphate is not lost at the phosphatidic acid phosphatase stage (Fig. 1) but, instead, it is the precursor of the phosphorus moiety of phosphatidylinositol. A number of cytidine diphosphate diglycerides were, therefore, synthesized (10) by a chemical procedure based on the method used previously for the synthesis of cytidine diphosphate choline (11). The resulting cytidine diphosphate diglycerides were readily isolated and purified by chromatography on silicic acid. With the aid of these pure synthetic materials, it was possible to demonstrate the reaction sequence shown in Fig. 3, leading to the formation of phosphatidylinositol in liver preparations.

The reaction of CDP-diglyceride with inositol could readily be followed by spectrophotometric methods since cytidine diphosphate diglycerides are insoluble in acid, whereas CMP, a product of the reaction, is soluble in acid. Since CMP has a characteristic absorption spectrum in the ultraviolet with a peak at 280 mμ, this proved to be a convenient and sensitive method for studying the formation of phosphatidylinositol.

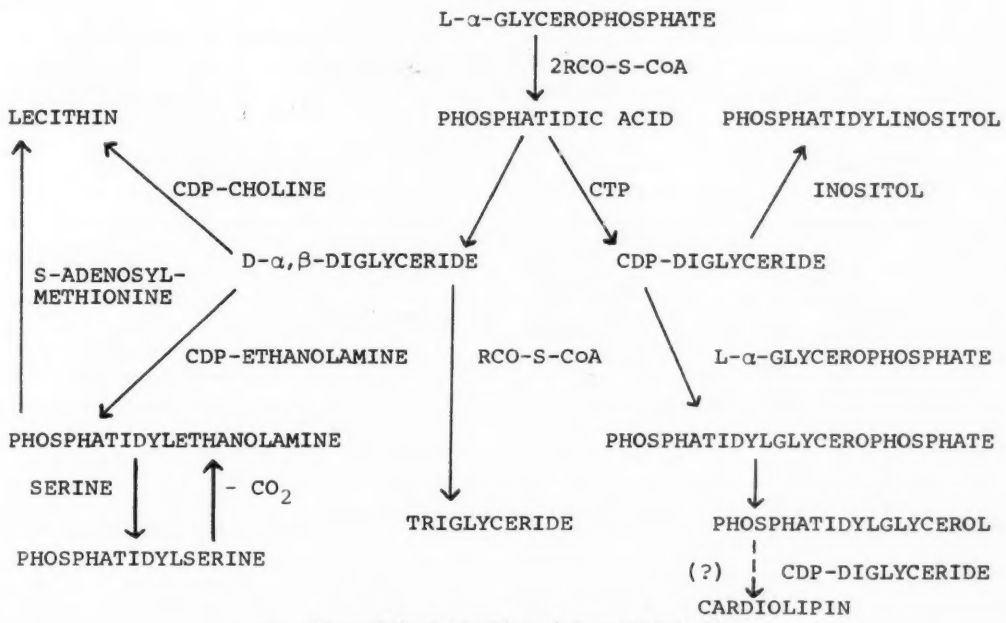

FIG. 1. Biosynthesis of glycerides and glycerophosphatides.

Agranoff and his collaborators (8) have suggested a somewhat different sequence of reactions for the formation of phosphatidylinositol, but it is possible that some of their results were complicated by the existence of an exchange reaction, demonstrated by Paulus and Kennedy (10) in liver preparations. The exchange reaction equilibrates the inositol portion of phosphatidylinositol with free inositol in the absence of sources of metabolic energy, apparently without a net change in the total amount of this phospholipid. Under certain experimental conditions, it may be stimulated by CMP, CDP-choline, or other cytidine nucleotides which may yield CMP on enzymatic hydrolysis. The exact relationship of this reaction to the de novo formation of phosphatidylinositol is not clear at present.

The biosynthesis of inositol-containing phospholipids in brain preparations has been studied by Thompson and his collaborators (12, 13). While some of the results are consistent with the formation of phosphatidylinositol, as shown in Fig. 3, other observations made by these workers suggest that the pattern of reactions in brain may be considerably more complicated, perhaps as a result of the functioning of enzyme systems leading to the formation of diphospho- and triphosphoinositides. Further work is urgently needed on the chemistry, as well as on the metabolism, of these more complex phosphoinositides.

Synthesis of polyglycerophosphatides. An enzymatic reaction between L-α-glycerophosphate and CDP-diglyceride was reported by Kiyasu, Paulus and Kennedy (14) in 1960. These workers have proposed that polyglycerophosphatides of the cardiolipin type may be formed by the reaction sequences shown in Fig. 4. The

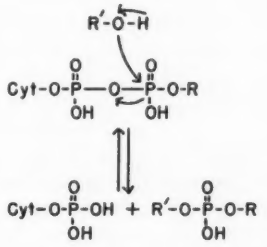

FIG. 2. Mechanism of formation of phosphodiester bonds.

first reaction is the formation of phosphatidylglycerophosphate by transfer of the phosphatidic acid portion of CDP-diglyceride to L-α-glycerophosphate. This product then undergoes enzymatic dephosphorylation to phosphatidylglycerol. The two steps can be differentiated, since the phosphatase is sensitive to inhibitors which attack sulfhydryl groups, while the enzyme catalyzing the formation of phosphatidylglycerophosphate is unaffected by these reagents.

Phosphatidylglycerol, itself, appears not to accumulate in animal tissues and it is, therefore, suggested that it may act as a precursor of cardiolipin by undergoing reaction with a second mole of CDP-diglyceride, as shown in Fig. 4. However, no direct evidence for the last step in this sequence has as yet been obtained.

Methylation of phosphatidylethanolamine. In 1953 Stekol (15) suggested that the transfer of the methyl group of methionine to ethanolamine in the formation of choline involves a phospholipid form of ethanolamine. Nyc and his collaborators (16) showed that a mutant strain of Neurospora crassa, which requires choline for optimal

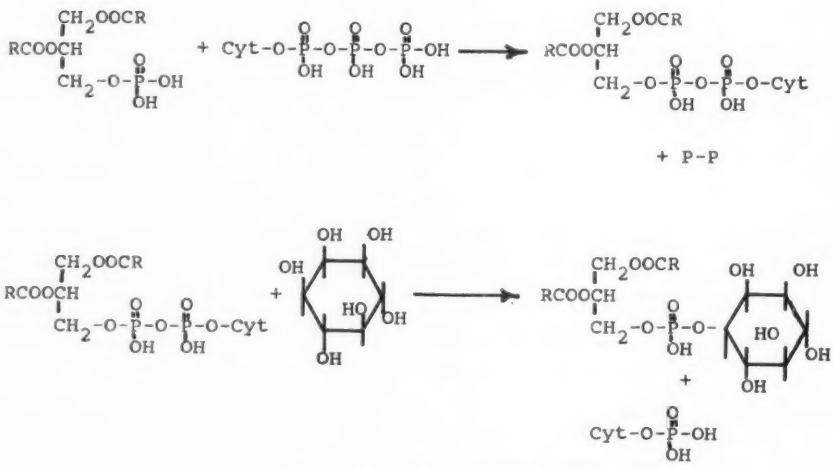

FIG. 3. Biosynthesis of phosphatidylinositol.

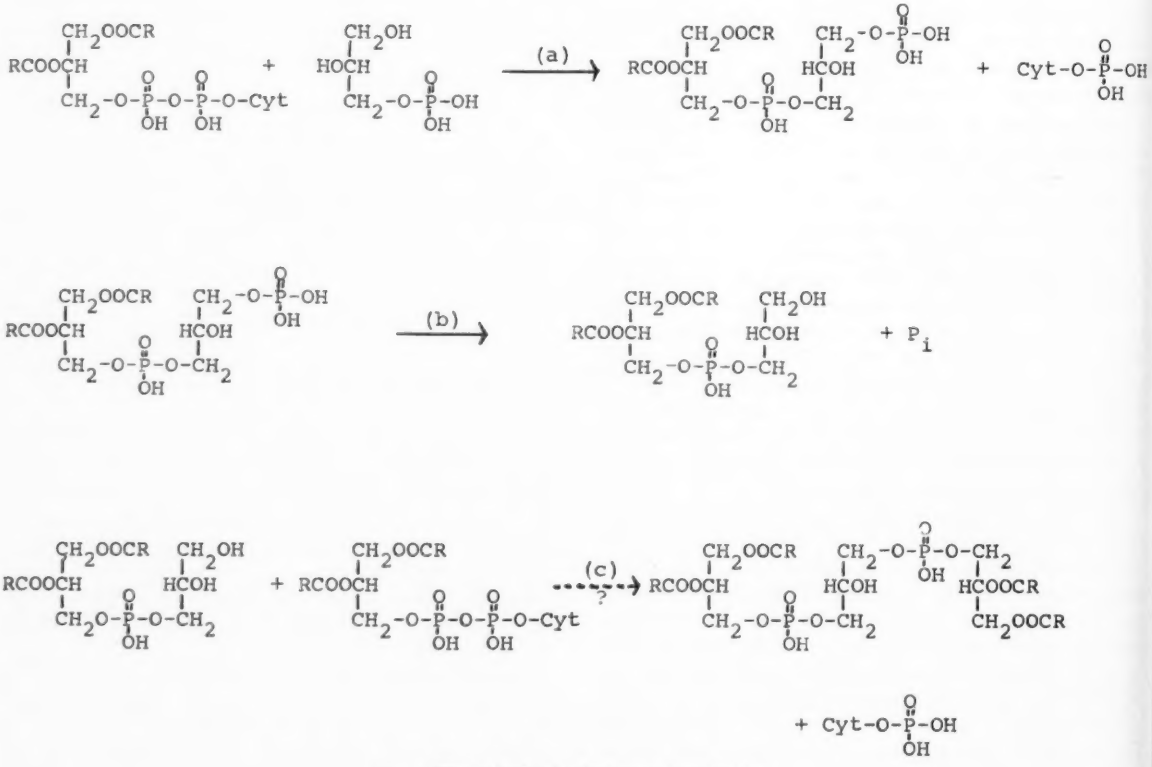

FIG. 4. Biosynthesis of polyglycerophosphatides.

growth, accumulates phospholipids containing mono- and dimethylaminoethanol when grown in the absence of choline. Crowder and Artom (17) found that dimethylaminoethanol could be converted to a phospholipid form in vitro, offering further support for the idea that phospholipids were involved in the methylation sequence.

Recent important studies of Bremer and Greenberg (3) have now provided direct evidence for the methylation of phosphatidylethanolamine to lecithin. The enzyme system required for these transformations is localized in the microsome fraction of liver, and utilizes S-adenosylmethionine as methyl donor. Phosphatidylethanolamine, phosphatidyl(N-methyl)ethanolamine and phos-

phatidyl(N,N-dimethyl)ethanolamine appear to be intermediates. No water-soluble methylated form of ethanolamine is directly involved. The pH optimum of the system is unusually high (pH 10–10.5).

Essentially similar results have been independently obtained by Udenfriend and his collaborators (18).

The methylation of phosphatidylethanolamine constitutes an alternative pathway for the biosynthesis of lecithin (Fig. 1), and it would be of interest to determine the amounts of lecithin synthesized in vivo by this route in comparison with the pathway involving choline, phosphorylcholine and cytidine diphosphate choline as intermediates. Bremer, Figard and Greenberg (19) have suggested that the methylation reactions may play an important role not only in the conversion of ethanolamine to choline, but in the conversion of choline itself to lecithin, by way of betaine, glycine and serine. However, the studies of Dawson (20) show that in vivo the phosphorylation of choline proceeds at a rapid rate in liver, sufficient to account for the overall synthesis of lecithin from orthophosphate, although this worker was careful to point out that the kinetics of the labeling of phosphorylcholine and lecithin did not fit perfectly with the precursor-product relationship postulated by Zilversmit (21), suggesting the possibility of alternative pathways. Furthermore, in another investigation of the labeling of various liver phospholipids from orthophosphate in vivo Dawson (22) found no evidence to suggest that the conversion of phosphatidylethanolamine to lecithin is the predominant reaction for the formation of the latter phospholipid. It should also be pointed out that the methylation of phosphatidylethanolamine appears to occur principally in liver. The activity of kidney tissue is quite feeble (3) while no significant methylation could be observed with brain enzymes. In contrast, the CDP-choline pathway is extremely active in kidney, which has a high rate of lecithin renewal in vivo, and Rossiter and collaborators (23) have clearly demonstrated the occurrence of the CDP-choline reactions in brain and nerve.

It seems probable that the relative contributions of the two pathways may be dependent upon the nutritional state of the animal. In animals on artificial diets very low in choline, the methylation reactions would be essential for the production of sufficient choline to meet the needs of the animal. However, in animals acutely deficient in dietary choline, the level of CDP-choline in the liver does not fall significantly below normal (24), suggesting that even in this extreme case the CDP-choline pathway is fully operative.

Acylation of lyso-lecithin. An enzyme which catalyzes the acylation of lyso-lecithin utilizing thioesters of coenzyme A has been described by Lands (25). This enzyme, which is present in microsomes of rat liver, is specific for lyso-lecithin, since it will not reacylate glycerophosphorylcholine. It does not require divalent cations, nor is it affected by chelating agents.

The occurrence of this acylating enzyme provides strong support for the concept of independent turnover of the fatty acid moieties of lecithin. A cycle may be visualized in which a fatty acid residue is removed from the lecithin molecule for oxidation or other utilization. The lyso-lecithin so formed may be further hydrolyzed yielding glycerophosphorylcholine, which then cannot be re-acylated, but is degraded to glycerophosphate and choline (26). Alternatively, the lyso-lecithin may react with the thioester of CoA to form lecithin once again.

The acylation of lyso-lecithin in this system is coupled to the release of free energy from ATP, since ATP is required for the formation of thioesters of CoA. It is thus to be distinguished from the reversal of hydrolysis catalyzed by lecithinases (27). It seems doubtful that the latter type of reaction can lead to a significant increase in ester bonds under physiological conditions.

Enzymatic formation and decarboxylation of phosphatidylserine. The enzymatic synthesis of phosphatidylserine has been investigated by Hübscher, Dils and Pover (28), who found that the conversion of free serine uniformly labeled with C^{14} to phospholipid in liver preparations could be stimulated by CMP under certain experimental conditions, but that the effects noted could not readily be fitted into a coherent pattern of biosynthesis. A much more striking effect of calcium ion in stimulating the incorporation of serine into lipid in the absence of added sources of metabolic energy was also observed by these workers, who suggested that it might be due to the reversible action of a calcium-stimulated phospholipase of the type that might cleave the serine moiety from phosphatidylserine.

Borkenhagen, Kennedy and Fielding (4) obtained evidence for the following sequence of reactions in cell-free preparations of liver:

(1) phosphatidylethanolamine + L-serine $\rightleftharpoons$ phosphatidylserine + ethanolamine

(2) phosphatidylserine $\rightarrow$ phosphatidylethanolamine + CO_2

sum: L-serine $\rightarrow$ ethanolamine + CO_2

These workers observed that both L-serine-3-C^{14} and ethanolamine-1,2-C^{14} are converted to lipid forms by rat liver homogenates in the presence of calcium ion and have shown that ethanolamine and serine compete for the same enzymatic site. Thus, unlabeled L-serine displaces labeled ethanolamine competitively from the enzyme surface and vice versa. The enzyme appears to be quite specific. D-Serine and choline, for example, are only feebly converted into lipid under these conditions and do not inhibit the enzymatic exchange of L-serine and ethanolamine.

Since a pathway for the de novo synthesis of phosphatidylethanolamine has already been established (29), reaction (1) may account for the formation of phosphatidylserine from simple precursors, utilizing the same reactions up to the point of formation of phosphatidylethanolamine.

Upon analysis of the labeled phospholipids derived from L-serine-3-C^{14} in their system, Borkenhagen et al. (4) found that a considerable part of the radioactivity

TABLE 1. *Enzymatic Decarboxylation of Phosphatidylserine*

Conditions of Incubation	$C^{14}O_2$ Produced, mμmoles
1. Complete	147
2. Omit toluene	32
3. Complete; boiled enzyme	0

Each tube contained EDTA (0.004 M), pyridoxal phosphate (0.001 M), dipalmitoyl-L-α-glycerophosphoryl-DL-serine-1-C^{14} (0.001 M) with a specific activity of 33,000 counts/min per micromole, and 0.31 ml of a suspension of rat liver mitochondria in isotonic sucrose containing 17.3 mg of protein per ml. The pH of the mitochondrial suspension was 6.5. No buffer was added. The final volume of the system was 0.50 ml. The tubes were incubated at 37° for 2 hr in special flasks provided with a center well containing 0.2 ml of 2 N KOH. The radioactive CO_2 was collected as $BaCO_3$, plated and counted with correction for self-absorption. In *tube 3*, the mitochondrial enzyme suspension was boiled for 5 min before addition to the flask. [From Borkenhagen, Kennedy and Fielding (4).]

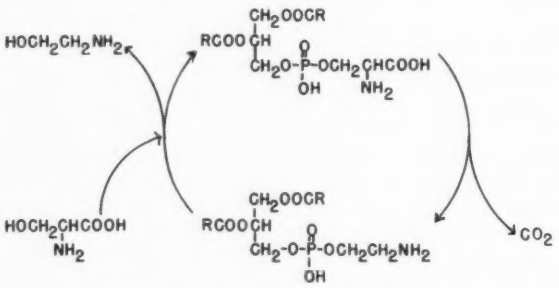

FIG. 5. Phospholipid cycle in decarboxylation of serine.

was present as phosphatidylethanolamine, rather than phosphatidylserine. This result, together with the previous studies of Wilson, Gibson and Udenfriend (30), and of Bremer, Figard and Greenberg (19), strongly suggested that a decarboxylation of phosphatidylserine was taking place. Accordingly, phosphatidylserine labeled with serine-1-C^{14} was synthesized by chemical procedures based on the methods of Baer and Maurukas (31). With the aid of this labeled substrate, a phosphatidylserine decarboxylase has been demonstrated in liver, brain and other tissues of the rat. A striking property of this enzyme is the requirement for organic solvents, such as toluene or octanol, for maximum activity (Table 1).

Reactions (1) + (2) constitute a metabolic cycle, in which phospholipids act as catalytic intermediates in the decarboxylation of L-serine, forming free ethanolamine and carbon dioxide, as shown in Fig. 5. It is important to note that the free ethanolamine released in this reaction cycle is not derived directly from L-serine, but from phosphatidylethanolamine. Previous attempts to demonstrate an active decarboxylase for free serine in animal tissues have met with failure, indicating that the 'phospholipid cycle' shown in Fig. 5 may be the principal mechanism for the production of ethanolamine from serine. This suggestion is strongly supported by the experiments of Luck and his collaborators (32), who reported in 1953 that the administration of large amounts of free serine to animals or to man leads to the excretion of free ethanolamine in the urine. Nemer and Elwyn (33, 34) showed that the concentration of free ethanolamine in the blood rises markedly shortly after the injection of serine, but, if serine-3-C^{14} is used, the free ethanolamine of the blood does not have a high specific radioactivity. These observations are entirely consistent with the mechanism shown in Fig. 5 in which the transient high concentration of free serine would lead to a displacement of ethanolamine from phosphatidylethanolamine. The appearance of free ethanolamine in the blood and the urine would be explained as, also, would the fact that the ethanolamine is not derived directly from serine.

The interconversion of phosphatidylethanolamine and serine in this cycle leads to significant changes in the physicochemical characteristics of the phospholipids, since it has been shown that phosphatidylserine is capable of binding cations at neutral pH, whereas phosphatidylethanolamine has little capacity to bind cations under these conditions (35). It is possible that metabolic function of these phospholipids may be mediated by this or similar reaction cycles.

BIOSYNTHESIS OF TRIGLYCERIDES

A scheme for the biosynthesis of triglycerides in liver was proposed in 1956 (36), in which glycerol, L-α-glycerophosphate, phosphatidic acid and D-α,β-diglyceride are successive intermediates (Fig. 6). More recent investigations have established that the principal features of the scheme also apply to the formation of triglyceride in intestinal mucosa (37–39) and adipose tissue (40, 41), with the important qualification that glycerokinase is absent from these tissues, being found only in liver, kidney and heart. For this reason, glycerol is not an effective precursor of glycerides in adipose tissue or in intestinal mucosa. L-α-Glycerophosphate needed for the formation of triglyceride is formed in these tissues by the reduction of dihydroxyacetone phosphate—a glycolytic intermediate. The esterification of fatty acid is, thus, linked to glycolysis—a fact which deserves serious

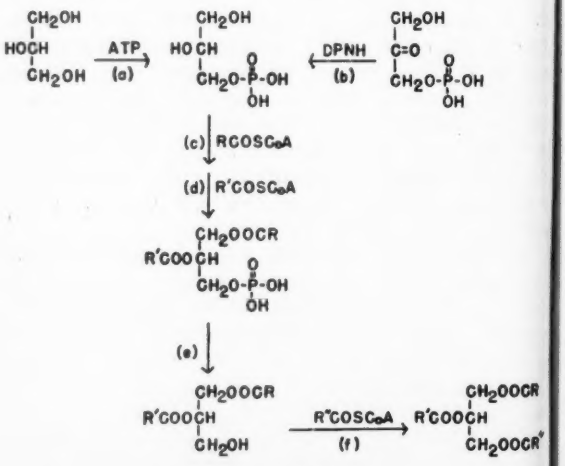

FIG. 6. Synthesis of triglycerides.

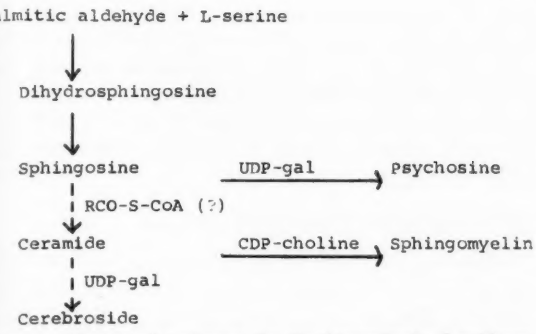

FIG. 7. Postulated pathways for the biosynthesis of sphingolipids.

consideration in efforts to unravel the complex interrelations of lipogenesis and carbohydrate metabolism, especially in adipose tissue.

A principal feature of the over-all scheme depicted in Fig. 1 is the relationship between the synthesis of triglycerides, on the one hand, and lecithin and phosphatidylethanolamine, on the other. Studies with isolated enzyme systems revealed that D-α,β-diglycerides are intermediates both in the synthesis of neutral fat and of these phospholipids. However, investigations on the specific localization of fatty acids in triglycerides showed a tendency for unsaturated fatty acid residues to predominate in the β position (42), although the work of Hanahan and his collaborators (43) seemed to show that the β position of egg lecithin was esterified predominantly with saturated fatty acids, since these were not cleaved by lecithinase A, an enzyme believed to attack specifically at the α' position. More recent work by Tattrie (44) and by Hanahan and his collaborators (45), has now disclosed that lecithinase A is specific for the β position of lecithin. The fatty acid pattern of triglycerides and of lecithin in rat liver (45) re-evaluated in the light of this information, is entirely consistent with the proposed scheme of biosynthesis.

D-α,β-Diglycerides, like the phosphatidic acids from which they are derived, constitute a family of compounds with fatty acids which may vary in chain length and degree of saturation. The enzyme which converts D-α,β-diglyceride to lecithin by reaction with CDP-choline (Fig. 1) has a pattern of specificity for diglycerides different from that of the triglyceride synthetase (46), with the result that the fatty acid composition of triglycerides and lecithin need not be identical. The same reasonsing applies to the fatty acid composition of other glycerophosphatides, such as phosphatidylinositol and of lecithin. Both are derived from the same phosphatidic acid pool, but by pathways catalyzed by different enzymes, which may have varying specificities for the fatty acid moieties of their substrates.

THE BIOSYNTHESIS OF SPHINGOLIPIDS

Information on the pathways of biosynthesis of sphingosine-containing lipids is at present quite incomplete. However, investigations in several laboratories on the formation of these important lipid components of brain and nerve are now under way, and the next few years will probably see considerable progress in this field. The pathways of biosynthesis presently known or postulated are shown in Fig. 7.

Biosynthesis of sphingosine. Isotope-tracer studies in vivo by Zabin and Mead (47), and Sprinson and Coulon (48) showed that carbon atoms 1 and 2 and the amino group of sphingosine are derived from serine, and carbon atoms 3 to 18 may be derived from acetate, probably via a 16-carbon intermediate. Ethanolamine was found not to be an effective precursor of sphingosine.

An enzyme system conforming to many of the requirements suggested by these findings in vivo was found by Brady and Koval (49, 50) in the brains of young rats. The following reactions have been suggested by these workers to account for the synthesis of sphingosine:

(3) palmityl CoA + TPNH → palmitic aldehyde + TPN + Co A
(4) palmitic aldehyde + L-serine → dihydrosphingosine + CO_2
(5) dihydrosphingosine → sphingosine + 2[H]

Zabin (51) has also observed the conversion of labeled serine to a sphingosine derivative, probably a ceramide, in a cell-free system from brain. Some of the properties of this system are similar to those described by Brady and Koval. Hoshishima, Vignais and Zabin (52) have more recently purified about 40-fold an enzyme which catalyzes the reduction of palmityl CoA to palmitic aldehyde. This enzyme, in contrast to that described by Brady et al., utilizes DPNH for the reduction, rather than TPNH.

Enzymatic synthesis of psychosine. The formation of psychosine (sphingosine galactoside) from free sphingosine and uridine diphosphate galactose, catalyzed by an enzyme present in microsomal preparations from guinea pig or rat brain, has been described by Cleland and Kennedy (53):

(6) UDP-galactose + sphingosine → sphingosine galactoside + UDP

Both sphingosine itself and dihydrosphingosine are effective acceptors for galactose in this reaction. A number of other compounds closely related to sphingosine also show some activity. In contrast, the enzyme is quite specific for uridine diphosphate galactose, since free galactose or galactose-1-phosphate is much less effective as a hexose donor.

Role of ceramides. As mentioned above, the labeled product derived from serine in Zabin's experiments (51) had properties resembling those of a ceremide. The exact sequence of reactions leading to this product remains unclear. Since the cofactor requirements described by Zabin are somewhat similar to those found by Brady and Koval (49) for the synthesis of free dihydrosphingosine, it is possible that free sphingosine is formed and then acylated to yield a ceramide. However, the exact stage at which the long-chain fatty acid amide is formed is still uncertain.

Enzymatic synthesis of sphingomyelin. Sribney and

Kennedy (54) have described some properties of an enzyme (phosphorylcholine-ceramide transferase) present in liver and other tissues, which catalyzes the synthesis of sphingomyelin according to the following equation:

(7) CDP-choline + ceramide → sphingomyelin + CMP

This enzyme exhibited a high degree of specificity both for CDP-choline and the acceptor ceramide. However, ceramides with the threo configuration of hydroxyl and amino group were much more reactive than those with the erythro configuration, although the naturally occurring sphingolipids appear to contain exclusively the erythro isomer. The explanation for this anomalous finding is not apparent, but it is possible that the threo ceramides may be able to penetrate more readily to the enzyme surface, under the conditions of incubation in vitro.

Cerebroside formation. Burton, Sodd and Brady (55) have shown that the microsomal fraction of brain derived from young rats contains the enzymes necessary for the incorporation of glucose or galactose into a neutral glycolipid fraction. Uridine diphosphate galactose was the most effective hexose donor. Chromatographic and infra-red spectral studies indicated that the radioactive glycolipid formed was a cerebroside, suggesting the following reaction:

(8) UDP-galactose + ceramide → cerebroside + UDP

However, efforts to establish the nature of the lipid acceptor in these experiments were unsuccessful. The assay procedures used would not have detected the intermediary formation of psychosine. For this reason, it is not clear whether a ceramide is formed and reacts with UDP-galactose to give a cerebroside, or whether psychosine is first formed and then later acylated to yield a cerebroside. It is possible that both pathways are operative and may lead to glycolipids of different structure, e.g., gangliosides or cerebrosides.

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Biosynthesis of saturated fatty acids

F. LYNEN

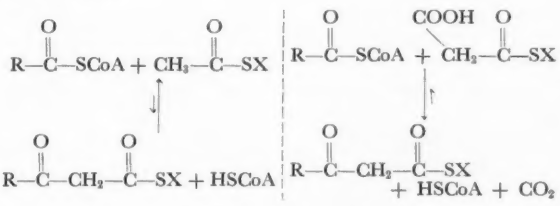
Max-Planck-Institut für Zellchemie, München, Germany

EVER SINCE THE CLASSICAL INVESTIGATIONS of Schönheimer (1), Sonderhoff (2), Rittenberg and Bloch (3) in the decade following 1935, it has been generally accepted that fatty acids are synthesized from acetate. After the discovery of the metabolically active form of acetate, acetyl CoA, had led to the isolation and characterization of the enzymes of the fatty acid oxidation cycle (4–6) it was an obvious assumption, that fatty acid synthesis from acetyl CoA could be achieved by the same enzymes, since each step in fatty acid degradation is reversible (Fig. 1).

There is, however, a serious difficulty underlying this assumption. The equilibrium of the thiolase reaction which catalyzes the condensation of acyl CoA with acetyl CoA to form the homologous β-ketoacyl CoA, does not favor the synthetic process (7). The concentration of the ketoacid derivative formed in this manner can only be very small under physiological conditions, as calculated from the known equilibrium constant (8). This difficulty could be circumvented, however, by assuming that the ketoacyl CoA formed is continuously removed by the subsequent reductive steps, thus shifting the equilibrium of the thiolase reaction in the direction of synthesis and effecting the synthesis of saturated acids. As a result of the discovery by Langdon (9) and by Seubert et al. (10) of a new enzyme in mammalian liver, which was able irreversibly to reduce CoA-thiolesters of unsaturated acids to the saturated ones with TPNH, it was possible to construct a system composed of purified enzyme components and electron donors such as DPNH and TPNH, which was able to synthesize fatty acids (10).

Following these experiments and similar experiments of Popják et al. (11) we felt we had succeeded in imitating a biological process. While we now know that other mechanisms for fatty acid biosynthesis exist, this scheme remains as a valid alternative or complimentary mechanism. Mitochondria, containing all the enzymes of fatty acid oxidation cycle, should be able to synthesize fatty acids in this manner. Wakil (12) has reported on a different mitochondrial system requiring ATP in addition to TPNH for the synthesis of fatty acids from acetyl CoA, although the implications of these new results await the details of the action of the ATP.

The equilibrium in the case of the malonyl-CoA system for the synthesis of fatty acids which Wakil (13) brought to light is quite different. The advantage gained in substituting malonyl CoA for acetyl CoA is very evident (14).



The condensation of acyl CoA with the thioester of malonic acid leads to the same β-ketoacyl derivative as with the thioester of acetic acid, but in this case the simultaneous liberation of CO_2 shifts the equilibrium toward synthesis. From a chemical standpoint, the reaction may be classified as an acylation of malonic ester (Fig. 2). The nucleophilic methylene group of malonyl CoA adds to the electrophilic carbonyl carbon of the thiolester, which is possibly further activated by an electrophilic center of the enzyme. The intermediate formed is converted to the ketoacyl derivative by the elimination of CO_2 and mercaptan. The primary product is the enol, which can subsequently tautomerize to the keto acid.

With respect to the malonyl CoA, formation by carboxylation of acetyl CoA, we now know that CO_2 activation occurs by the following scheme (Fig. 3) (13–17).

The reaction belongs to the class of carboxylation processes, in which biotin participates as the coenzyme (17, 18). We may assume, that the carboxylation follows the same mechanism as elucidated for the carboxylation of β-methylcrotonyl CoA (19, 20) implying that the "active CO_2" is in the form of N-carboxybiotin. This is shown symbolically in the reaction sequence.

This scheme must be considered with some reservation, because it is supported only by the demonstration that biotin is the cofactor of the enzyme (18, 21) and by the balance of the ATP cleavage (17 and personal communication, Wakil). Further details of the mechanism of malonyl CoA formation will doubtlessly prove to be interesting. For example, alternative pathways leading to malonyl CoA may exist. Reports from various laboratories (9, 22–24), including Dr. Numa (25) in my laboratory with rat liver extracts, have shown that isocitrate is required for the incorporation of acetate into fatty acids. The isocitrate effect cannot, however,

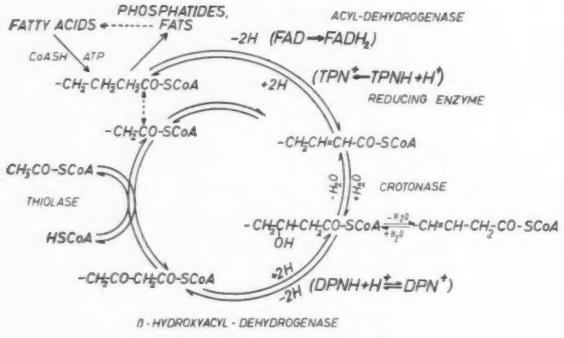


FIG. 1. The "fatty acid cycle."

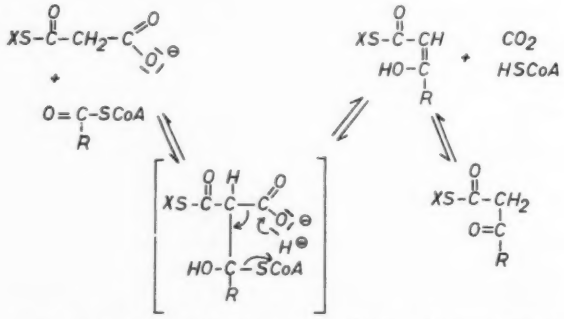


FIG. 2. Mechanism of the formation of β-ketoacids from malonyl thioester.

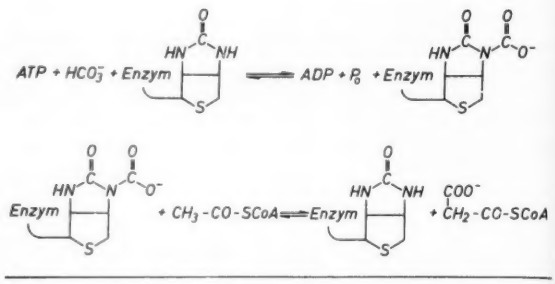


FIG. 3. Mechanism of the carboxylation of acetyl-CoA.

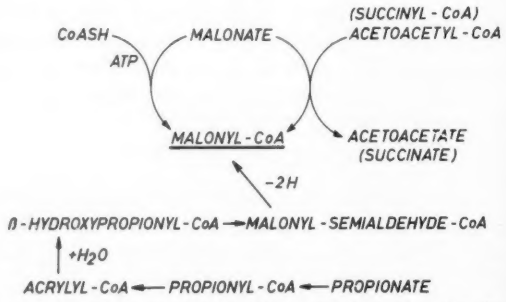


FIG. 4. Metabolic reactions leading to the formation of malonyl CoA.

be explained by its ability to give rise to bicarbonate or reduced TPN and must remain for the moment unexplained (25).

In addition to this, malonyl CoA may be formed from precursors other than acetyl CoA. Hayaishi (26) has reported on the ATP dependent activation of malonic acid with CoA, and Beinert and Stansly (27), as well as Menon and Stern (28), have observed a transfer of CoA from acetoacetyl or succinyl CoA to malonate. It is not clear whether the latter reaction is of physiological significance or whether it is merely the outcome of the rather broad specificity of the well-known acetoacetyl-succinate-thiophorase (Fig. 4). The participation of these reactions also depends on the level of free malonic acid formed in normal metabolism.

Another pathway to malonyl CoA was discovered during investigations with *Clostridium kluyveri* extracts conducted by Vagelos (29) in Stadtman's laboratory on the degradation of propionic acid via acrylyl CoA and β-hydroxy propionyl CoA to malonyl-semialdehyde CoA, which can be, in turn, further dehydrogenated by TPN to form malonyl CoA (Fig. 4).

Let us now turn to the second phase of fatty acids synthesis, which is the conversion of malonyl CoA to higher fatty acids. We will refer to the enzyme system, which is responsible for these reactions, as "fatty acid synthetase." It behaves as a single enzyme during the fractionation and is widely distributed in animal tissues (30) particularly liver (25, 30–32), brain (33), adipose tissue (34), and lactating mammary glands (30). Highly active preparations may also be obtained from yeast (8, 14). Investigations of the catalytic nature of the purified enzyme, carried out in various laboratories (8, 31–35), support the following equation for fatty acid synthesis from malonyl CoA:

$$(1)\quad CH_3\text{—}CO\text{-}SCoA + n\ \underset{|}{\overset{COOH}{CH_2}}\text{—}CO\text{-}SCoA + 2n\ TPNH + 2n\ H^+ \rightarrow CH_3\text{—}(CH_2\text{—}CH_2)_n\text{—}CO\text{-}SCoA + n\ CO_2 + n\ HSCoA + 2n\ TPN^+ + n\ H_2O \text{ (for palmityl CoA: } n = 7)$$

When DPNH is substituted for TPNH, the rate decreased by about 75%. In our hands, the purified yeast enzyme yields palmityl and stearyl CoA as the principal products (8, 35), accompanied by minor quantities of shorter, even numbered fatty acids. Enzymes derived from animal sources yield mainly free palmitic acid (31–34, 36, 37). According to Porter and Long (38), palmityl CoA is the primary product and is subsequently cleaved by a deacylase present in impure preparations. A very characteristic property of the synthetase is the absolute requirement for acetyl CoA, which is incorporated into the final fatty acid product. The acetyl CoA acts as a "primer" in fatty acid synthesis. It is incorporated into the "tail" end of the fatty acid, which is formed by the successive addition of C_2-units derived from malonyl CoA to the "primer" acyl CoA (Fig. 5). A large number of saturated acyl CoA such as propionyl,

butyryl, hexanoyl CoA, and, in the case of the yeast enzyme, even palmityl CoA, can replace the acetyl CoA as the primer (8, 31, 35).

Obviously, when propionyl CoA is used as the primer, odd numbered fatty acids are formed. Branched chain acyl CoA, such as isovaleryl CoA, can also be used as a primer, in which case fatty acids, branched at the tail end, are produced (39). In contrast, the intermediate compounds which occur in the course of fatty acid degradation such as α,β-unsaturated, β-hydroxy and β-ketoacyl CoA derivatives, have been tested by Wakil and Ganguly (31) and by us (8, 35), and have been shown to be ineffective as primers for fatty acid synthesis from malonyl CoA. Consequently, these compounds are not intermediates in the course of fatty acid synthesis.

To explain this observation, Wakil and Ganguly (31) suggested a mechanism of fatty acid synthesis as shown in Fig. 6. An essential part of their proposed scheme is the formation of dicarboxylic acids intermediates, which are decarboxylated after reduction to alkylmalonic acid derivatives. The saturated acyl CoA thus formed condenses, in turn, with a second molecule of malonyl CoA. As can be seen, the saturated acyl CoA would be an intermediate, but not the substituted derivatives of the β-oxidation sequence, which is in complete agreement with the experimental observation. On the other hand, from the standpoint of energetics, such a scheme cannot take advantage of the decarboxylation of malonyl CoA for a synthetic reaction. We felt this to be a serious objection to the scheme.

After considerable effort in collaboration with Ingrid Kessel and Dr. Hermann Eggerer (35), we were able to get some insight into the secrets of this process. The first inkling of the direction which we were to follow came from the observation, that fatty acid synthesis can be inhibited by sulfhydryl blocking agents, such as iodoacetamide or arsenite (14). At .01 molar concentration, the enzyme was inhibited completely by both agents, and even with 10^{-4} molar concentration, some inhibition is observed (8). In view of this sensitivity, we routinely added thiols, such as cystein or glutathione, to the incubation mixtures, which resulted in a stimulation of fatty acid synthesis. In order to explain the stimulation by thiols and the inhibition by sulfhydryl binding agents, we assumed that the enzyme itself contains SH-groups, which are indispensible for the catalytic effect (8, 35) [cf Brady et al. (40)]. This fact, taken together with our repeated inability to demonstrate any free intermediates in the synthetic process, leads us to the working hypothesis that the actual mechanism involves a transfer of the

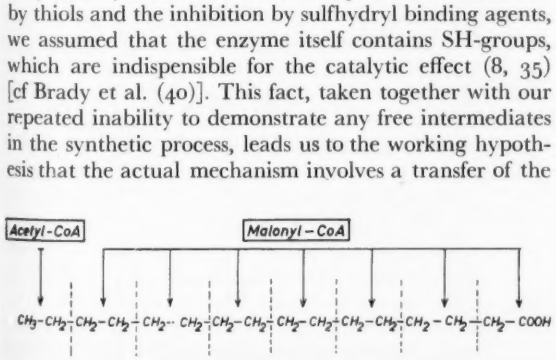


FIG. 5. Origin of the carbon atoms of palmitic acid.

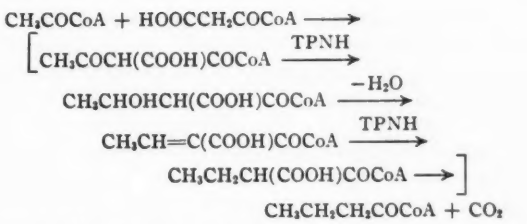
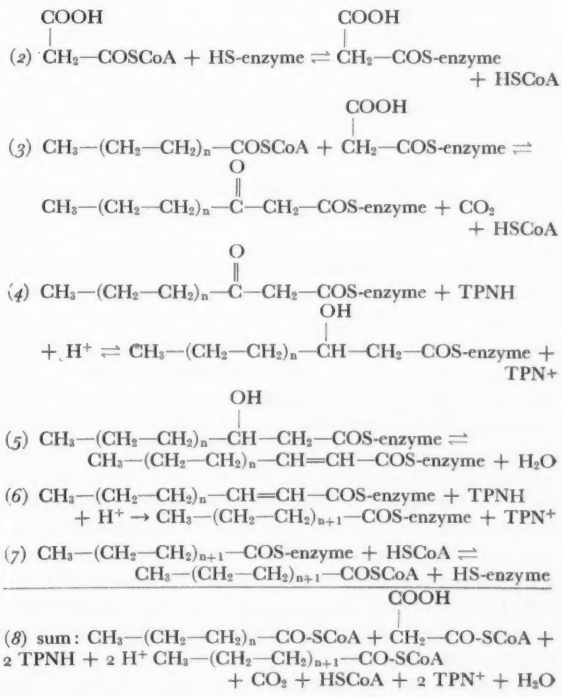


FIG. 6. Scheme of fatty acid synthesis by Wakil and Ganguly.

acyl portion of the coenzyme A esters to the sulfhydryl groups on the enzyme itself. We have obtained experimental evidence to support the following reaction scheme (eq. 2–7):


(2) CH2—COSCoA + HS-enzyme ⇌ CH2—COS-enzyme + HSCoA
(3) CH3—(CH2—CH2)n—COSCoA + CH2—COS-enzyme ⇌ CH3—(CH2—CH2)n—C(=O)—CH2—COS-enzyme + CO2 + HSCoA
(4) CH3—(CH2—CH2)n—C(=O)—CH2—COS-enzyme + TPNH + H+ ⇌ CH3—(CH2—CH2)n—CH(OH)—CH2—COS-enzyme + TPN+
(5) CH3—(CH2—CH2)n—CH(OH)—CH2—COS-enzyme ⇌ CH3—(CH2—CH2)n—CH=CH—COS-enzyme + H2O
(6) CH3—(CH2—CH2)n—CH=CH—COS-enzyme + TPNH + H+ → CH3—(CH2—CH2)n+1—COS-enzyme + TPN+
(7) CH3—(CH2—CH2)n+1—COS-enzyme + HSCoA ⇌ CH3—(CH2—CH2)n+1—COSCoA + HS-enzyme
(8) sum: CH3—(CH2—CH2)n—CO-SCoA + CH2—CO-SCoA + 2 TPNH + 2 H+ CH3—(CH2—CH2)n+1—CO-SCoA + CO2 + HSCoA + 2 TPN+ + H2O

Thus fatty acid synthesis is accomplished through the repetition of a cycle of these six consecutive reactions. The process is visualized as being initiated by the transfer of the malonyl residue from malonyl-CoA to the enzyme to form a malonyl-S-enzyme (eq. 2). The next step is the most distinguishing point in this scheme: the condensation of the saturated acyl CoA with the malonyl enzyme to form the β-ketoacyl enzyme (eq. 3). The accompanying decarboxylation merely adheres to the principle discussed earlier. It is significant that the condensation product is a β-ketoacyl enzyme rather than the β-ketoacyl coenzyme A. This is important from the biosynthetic point of view, since otherwise the thermodynamic advantage gained by the decarboxylation of malonyl CoA would be lost through the thiolytic cleavage of acetoacetyl CoA catalyzed by the enzyme thiolase.

After condensation, there follows a stepwise conversion involving first a reduction to the β-hydroxyacyl enzyme (eq. *4*), then a dehydration to the dehydroacyl enzyme (eq. *5*), and, finally, a reduction to the saturated acyl enzyme (eq. *6*). The saturated acyl group thus formed can be transferred back to coenzyme A (eq. *7*).

In these six steps, the carbon chain of the acyl CoA, with which we started, has been increased by two carbon atoms in a reaction sequence requiring one mole of malonyl CoA and two moles of TPNH (eq. *8*). This elongated acyl CoA then reacts with malonyl enzyme in a new condensation sequence. It is also possible that the acyl CoA is not a required intermediate in the reactions which result in the elongation of the carbon chain, but rather that the acyl-S-enzyme can directly condense with another malonyl-S-enzyme. The exclusion of the acyl CoA derivates could be facilitated if more than one sulfhydryl is present at the active site of the enzyme. However, the transfer back to the CoA must at some point be an integral part of the reaction sequence since the final product is always an acyl CoA.

The first experimental support for this scheme was the identification of the intermediate formation of a β-keto-acyl-enzyme, namely acetoacetyl-enzyme (8, 35). When 1-C^{14}-labeled acetyl CoA and malonyl CoA were incubated with stoichiometric amounts of the purified yeast enzyme and the protein was precipitated by the addition of trichloroacetic acid, it contained bound radioactive acetoacetate, which could be released by mild alkaline hydrolysis and which yielded radioactive acetone upon decarboxylation (Fig. 7). When the washed precipitated acyl protein was treated with potassium borohydride, and then subjected to alkaline hydrolysis, radioactive β-hydroxybutyric acid was isolated. In this case, we determined the position of the radioactive carbon by the van Slyke (41) oxidation procedure. The acetone thus formed contained all the radioactivity, the CO_2 being practically free of radioactive carbon (Fig. 7). This result proved unequivocally that the condensation of acetyl CoA and malonyl CoA had occurred in the manner depicted in Fig. 7.*

I learned at the present Federation Meetings that during the past year our finding of β-ketoacyl enzyme has been confirmed by Alberts and Vagelos (42) with an enzyme system from *Clostridium kluyveri*. We have since been able to further unravel some of the details of this complicated enzymic process. First, we could prove that the synthetase from yeast also catalyzes the condensation of higher acyl CoA compounds with malonyl CoA to form the corresponding β-ketoacyl enzymes. Thus, β-ketodecanoyl-enzyme is formed when 1-C^{14}-octanoyl CoA is incubated with malonyl CoA and stoichiometric amounts of enzyme. The reaction product can be hydrolyzed to the free ketoacid and has been identified by decarboxylation to radioactive methylheptyl ketone. It turned out, furthermore, that both reactions were reversible in agreement with thermodynamic calculations. This is to be expected from the work of Vagelos (43, 44), who discovered that *Clostridium kluyveri* extracts catalyze an acyl CoA dependent exchange reaction between labeled bicarbonate and the free carboxyl group of malonyl CoA. Using Vagelos' technique (44), we demonstrated that the fatty acid synthetase from yeast also catalyzes the incorporation of labeled CO_2 into malonyl-CoA when a saturated acyl CoA derivative, for example caprinyl CoA, is added (eq. *9* and *10*).

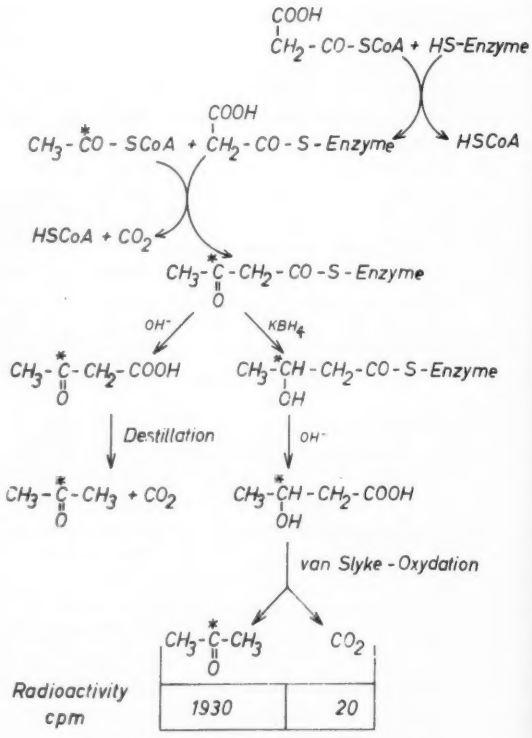

COOH
CH2 - CO - SCoA + HS-Enzyme
HSCoA
CH3 - CO - SCoA + CH2 - CO - S - Enzyme
HSCoA + CO2
CH3 - C - CH2 - CO - S - Enzyme
OH⁻
KBH4
CH3 - C - CH2 - COOH
CH3 - CH - CH2 - CO - S - Enzyme
OH
Destillation
OH⁻
CH3 - C - CH3 + CO2
CH3 - CH - CH2 - COOH
OH
van Slyke - Oxydation
CH3 - C - CH3
CO2
Radioactivity cpm
1930
20

FIG. 7. Identification of acetoacetyl enzyme.

(9) $$\overset{*}{\text{COOH}}\text{—}\underset{\underset{\text{O}}{\|}}{\text{CH}_2\text{—C—SCoA}} + \text{HS-enzyme} \rightleftharpoons \overset{*}{\text{COOH}}\text{—}\underset{\underset{\text{O}}{\|}}{\text{CH}_2\text{—C—S-enzyme}} + \text{HSCoA}$$

(10) $$\text{R—}\underset{\underset{\text{O}}{\|}}{\text{C—SCoA}} + \overset{*}{\text{COOH}}\text{—}\underset{\underset{\text{O}}{\|}}{\text{CH}_2\text{—C—S-enzyme}} \rightleftharpoons \text{R—}\underset{\underset{\text{O}}{\|}}{\text{C—CH}_2\text{—}}\underset{\underset{\text{O}}{\|}}{\text{C—S-enzyme}} + \overset{*}{\text{CO}}_2 + \text{HSCoA}$$

* These results have been also presented at the Rudolf Schoenheimer Lecture in New York (April, 1960).

But since the rate of exchange observed was only about 20% of the rate of fatty acid synthesis, we can conclude that the equilibrium of the condensation lies on the side of β-ketoacyl formation, as discussed before (page 941).

The next step was to try to isolate the β-ketoacyl enzyme in its native form. We did not succeed, however, due to a slow hydrolysis reaction yielding the free keto-acid (8). Compared with the rate of fatty acid synthesis, the rate of hydrolysis is 3,000 times slower and is, therefore, negligible with regard to fatty acid synthesis, but in experiments requiring large amounts of enzyme, the hydrolysis does not permit the isolation of the native β-keto acyl enzyme.

In order to demonstrate the manifold catalytic activities attributed to the enzyme, we were able to utilize the earlier experience gained, by replacing the complicated coenzyme A residue by N-acetyl cysteamine or pantetheine (45, 46). For the problem under discussion, this technique proved to be tremendously useful, so that we are now able to demonstrate every single reaction step in the sequence independent of preceding or subsequent reactions. The affinity of these model compounds is generally rather low compared with the natural substrated. This can be circumvented by employing high concentrations of the model substrates.

The observation that purified yeast-synthetase is able to utilize acetyl and malonyl pantetheine in place of acetyl and malonyl CoA enabled us to investigate the initial malonyl transfer from CoA to enzyme sulfhydryl (8). In accord with the proposed mechanism, we were forced to conclude that malonyl pantetheine transfers the malonyl group to the enzyme in the same way as malonyl CoA (eq. *11*). Consequently the enzyme should catalyze the transfer of the malonyl residue from malonyl CoA to pantetheine (eq. *12*).

(*2*) malonyl CoA + enzyme $\rightleftharpoons$ CoA + malonyl-enzyme

(*11*) malonyl-enzyme + pantetheine $\rightleftharpoons$ enzyme + malonyl-pantetheine

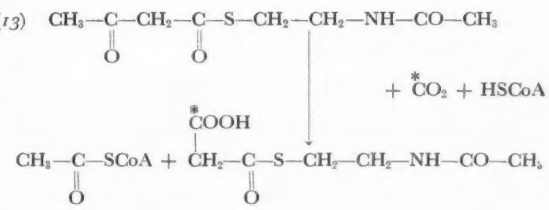
---

(*12*) malonyl-CoA + pantetheine $\overset{\text{(enzyme)}}{\rightleftharpoons}$ CoA + malonyl-pantetheine

In the experiment presented in Fig. 8, various amounts of purified synthetase were incubated with 2-C^{14}-malonyl CoA and pantetheine and the formation of labeled malonyl pantetheine was determined. The enzyme proved to be a very efficient malonyl-transacylase, catalyzing the transfer of malonate at more than four times the rate required for the over-all fatty acid synthesis (8). In addition to this, Hagen in my laboratory was able to demonstrate that the ratio between synthetase and transacylase-activity remained constant in the course of a 200-fold purification of the enzyme. Thus it is evident that this transacylase activity is intimately associated with the synthetase and is not a separate entity.

I shall turn now to the condensation step. The participation of the β-keto acyl thiol derivative in the condensation can be demonstrated by studying the reverse reaction with radioactive bicarbonate (eq. *13*).

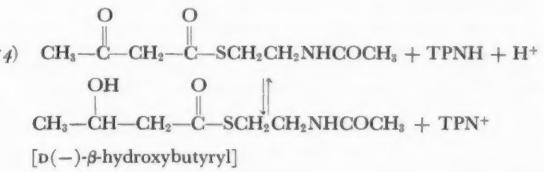
(*13*)



The incorporation of radioactive CO_2 into S-malonyl-N-acetylcysteamine, which could be isolated and identified, is dependent upon the addition of the β-ketoacid thiolester. This can be seen in Fig. 9, which shows that the fixation of CO_2 into the malonyl moiety is directly related to the concentration of S-acetoacetyl-N-acetyl-cysteamine, which can be utilized by the enzyme. Rather high concentrations of the synthetic model substrate are required in this reaction.

This is also true in the assay for the next step, the reduction of the β-ketoacyl derivatives by TPNH (eq. *14*):

(*14*)

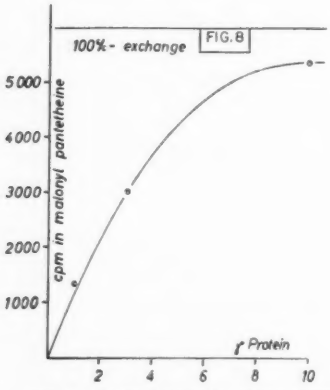


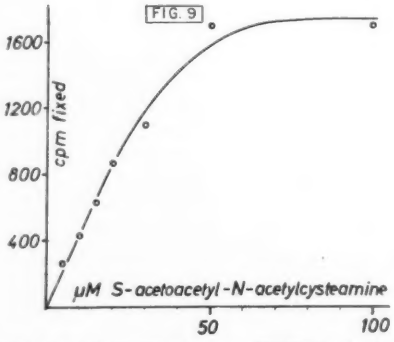
[D(−)-β-hydroxybutyryl]

In Fig. 10, the dependence of the rate of TPNH-oxidation on the concentration of the model substrates acetoacetyl-CoA and S-acetoacetyl-N-acetyl-cysteamine is shown. As can be seen, the characteristic Michaelis-Menten curves are not obtained at the level of synthetic substrates employed. However, by the graphical extrapolation, using the method of Lineweaver and Burk (47), a Michaelis constant of 1×10^{-2} [moles substrate per liter] was estimated for both substrates.

We can compare the reduction of acetoacetyl CoA or S-acetoacetyl-N-acetyl-cysteamine by the synthetase enzyme with the β-hydroxyacyl-dehydrogenase operating in fatty acid degradation. There are, however, several points of difference. One is the low affinity of the synthetase for acetoacetyl CoA, while the corresponding enzyme in the oxidation sequence has a high affinity for this substance (48). This would be expected, since the CoA derivative is no better a model than the cysteamine derivative for the synthetic enzyme. Another point of difference is the specificity of the synthetase for TPNH and the D(-)-β-hydroxybutyryl derivative in contrast to DPNH and L(+)-β-hydroxybutyryl CoA (48).

The same difference in stereospecificity was also found in the subsequent enzymic reaction, the dehydration of the β-hydroxy derivative to the α,β-unsaturated compound. This step was demonstrated by the use of S-β-hydroxybutyryl-N-acetyl-cysteamine as a substrate and by following the characteristic absorption of dehydroacyl thioesters at 263 mμ, found by Seubert in my laboratory (45) (eq. *15*).





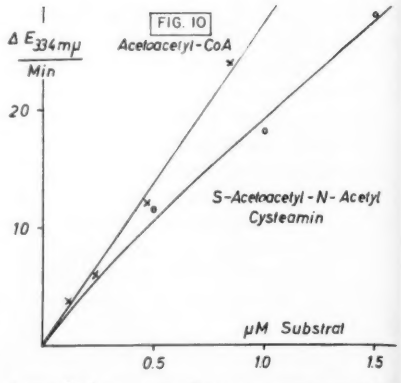


FIG. 8. Malonyl transfer between malonyl CoA and pantetheine. 100 μM K-phosphate, pH 6.5, 15 μM cysteine, 0.115 μM 2-C^{14}-malonyl CoA (=52,000 cpm/μM) 1.11 μM pantetheine in a total volume of 1.5 ml were incubated 10 min at 23 C. After the addition of 0.1 ml 2 N H_2SO_4 to stop the reaction, malonyl pantetheine and malonyl CoA were separated by phenol extraction and chromatography on Dowex 2. The malonyl pantetheine is eluted from the Dowex column by M formic acid.

FIG. 9. Dependence of $C^{14}O_2$-incorporation into S-malonyl-N-acetyl-cysteamine on the concentration of S-acetoacetyl-N-acetyl-cysteamine. The incubation mixtures contained 50 μM Tris-buffer, pH 7.5, 5 μM cysteine, 5 μM ethylenediamine tetraacetate, 2 μM $KHC^{14}O_3$ (2 × 10^5 cpm/μM), 0.15 μM CoA, 1.05 mg synthetase protein (sp. act. 150) and S-acetoacetyl-N-acetyl-cysteamine, as designated in the figure. Total volume: 0.5 ml. After 15 min incubation at 30 C the mixture was acidified with 0.5 ml 5 N acetic acid and the fixed radioactivity was measured.

FIG. 10. Dependence of the initial rate of TPNH oxidation on the concentration of model acetoacetyl thioesters. 1 cm cuvettes contained 100 μM K-phosphate, pH 6.5, 15 μM cysteine, 0.12 μM TPNH, 9.4 γ synthetase protein and S-acetoacetyl-N-acetyl cysteamine or acetoacetyl CoA, as designated in the figure. Total volume: 1.5 ml. Temp.: 20 C. The rate of TPNH disappearance was measured at 334 mμ.

(15) $CH_3—\underset{\text{OH}}{\underset{|}{CH}}—CH_2—\underset{\text{O}}{\overset{\|}{C}}—S—CH_2—CH_2—NH—CO—CH_3 \rightleftharpoons$

[D(−)-β-hydroxybutyryl-]

$$\begin{matrix} CH_3 & & H \\ & C{=}C & \\ H & & C—S—CH_2CH_2NHCOCH_3 + H_2O \\ & & \| \\ & & O \end{matrix}$$

In this way, it was observed that only the D-β-hydroxybutyryl thioester was dehydrated, in contrast to crotonase, which was shown by Stern and Del Campillo (49) to be specific for the L-compound. Furthermore, crotonase does not react with the model N-acetyl-cysteamine derivatives, even at high substrate concentrations (unpublished experiments). With the synthetase S-crotonyl-N-acetyl cysteamine is formed. The isolated product could be identified by its characteristic ultraviolet-absorption, which is quite different from a spectrum of an isocrotonyl-thioester, as observed by Stern and Del Campillo (49).

S-crotonyl-N-acetyl-cysteamine was used as a substrate for the fifth step in the reaction sequence. The reduction to the butyryl-derivative was followed spectrophotometrically by way of the consumption of reduced TPN. Before going into these experiments, I want to explain briefly the purification procedure employed in the isolation of the synthetase from yeast, which was performed by Ingrid Kessel and Dr. Eckehard Lorch in my laboratory. The procedure is summarized in Table 1. By repeated fractional precipitation with ammonium sulfate, adsorption on calcium phosphate gel, removal of

TABLE 1. *Purification Procedure of Fatty Acid Synthetase from Yeast*
(Extract from 200 g baker's yeast)

Fraction	Protein, mg	Enzyme milliunits*	Specific Activity, mu/mg
1. Yeast extract	35,300	(141,250)	4
2. First $(NH_4)_2SO_4$ fractionation (35–50)	6,740	154,380	23
3. $Ca_3(PO_4)_2$-gel eluate	495	76,250	154
4. Second $(NH_4)_2SO_4$ fractionation (35–50)	179	56,250	314
5. Supernatant of alumina Cγ	103	45,500	448
6. Chromatography on hydroxyapatite	40	31,250	775

* One milliunit (mU) of enzyme catalyzes the incorporation of 1 mμM malonyl CoA (equiv. the oxidation of 2 mμM TPNH) per minute at pH 6.5 and 20 C.

impurities by alumina gel, and column chromatography on hydroxyapatite, the enzyme of the crude yeast extract could be purified about 200-fold. A further concentration of the enzyme was achieved by sedimentation of the total activity in the centrifuge at 100,000 × *g* for 5 hr.

The pellet formed had a striking yellow color, which suggested a high flavin content. We have now succeeded in proving that flavin mononucleotide is actually responsible for the yellow color, and that this nucleotide functions in the hydrogen transfer to the double bond in the second reductive step of fatty acid synthesis (Fig. 11). The flavin can be separated from the enzyme by treatment with acid ammonium sulfate solution, as de-

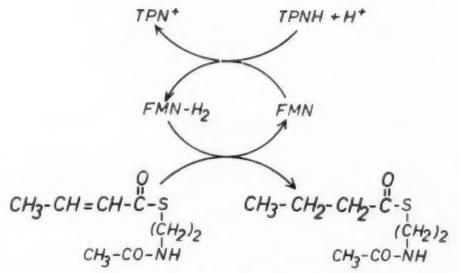


FIG. 11. Participation of FMN in the reduction of the crotonyl moiety by TPNH.

scribed by Warburg and Christian (50). The colorless protein precipitated in this procedure is almost completely inactive, but it can be reactivated by the addition of flavin mononucleotide. In contrast to the corresponding fatty acid degradation enzyme (51, 52), flavin adenine dinucleotide is largely ineffective as a coenzyme.

Definite proof for the participation of this flavoprotein in fatty acid synthesis was established by the fact that the addition of flavin mononucleotide also reconstituted the over-all process to a certain extent. Table 2 contrasts the specific enzyme activities of the acid-treated preparation in the presence and absence of FMN. The addition of the flavin stimulated the crotonyl reduction and the over-all process of fatty acid synthesis from acetyl-CoA and malonyl CoA, but was without effect on the other enzymic reactions. The degree of reactivation was identical for the crotonyl reduction and the over-all reaction as documented by the last column.

Examination of the values of the first column shows the specific activities of the intact enzyme for the various enzymic reactions. It is apparent that the rate of some of the reactions with the model substrates is slower than the rate of the over-all process with the natural substrates. This is, however, not surprising, inasmuch as our systematic studies on the enzymes of fatty acid degradation had shown (46) that the natural substrates exceed model compounds, not only in respect to their affinity for the enzymes, but also in respect to their turnover number. For these reasons, we cannot compare the rates observed with the model substrates with the rates observed with the natural substrates, which, in this case, are the various acyl enzymes. This conclusion is substantiated for example by the experiments on the cleavage and recombination of the crotonyl-reductase activity, which demonstrate that this enzyme must participate in the over-all process.

These experiments yielded still another finding which, to our mind, seems to be of utmost importance. Various single activities were damaged by the acid treatment to remove the FMN from the synthetase. The condensation reaction, as measured by the exchange of labeled CO_2 with the free carboxyl group of malonyl CoA (eq. 9 and 10), is most impaired. But, as it may be seen from the third column (table 2), one third of the original activity is retained. The fact that the over-all rate is depressed

TABLE 2. *Effect of FMN Removal on Enzyme Activities*

	SPECIFIC ACTIVITY (mμM SUBSTRATE CONSUMED/mg PROTEIN/MIN)				
TYPE OF REACTION	BEFORE SPLITTING	AFTER SPLITTING			REACTIVATION-FACTOR
		-FMN	+FMN	% OF ORIGINAL ACTIVITY	
TOTAL SYSTEM	270	4.2	34.2	12.7	8
ACETOACETYL-REDUCT.	360	193	187	51.8	—
β-HYDROXYBUTYRYL-DEHYDRATION	47.5	49	47	99	—
CROTONYL-REDUCT.	31.6	3.2	25.7	81	8
MAL-CoA $\rightleftharpoons$ $^*CO_2$	52	14.7	18.8	36	—
MALONYL-TRANSFER	1260	1250	1290	102	—

The splitting of FMN from the protein was performed as follows: 20 mg synthetase protein in 2.5 ml 32% aqueous ammonium sulfate solution 0°C was acidified to pH 3.95 with 0.1 ml 2 M acetic acid. The FMN was adsorbed onto charcoal (20 mg). The charcoal and the precipitated protein was centrifuged and the protein solubilized with 0.1 M Na-pyrophosphate, pH 8.3. This protein solution was used for the various enzyme assays.

When FMN was added the concentration in the assay was 0.007 μM/ml.

Total system of fatty acid synthesis cf. (8); *malonyl transfer reaction* cf. Fig. 8; *acetoacetyl thioester reduction* cf. Fig. 10; *dehydratation*: cuvettes (d = 0.5 cm) contained 500 μM Tris-buffer, pH 7.5, 20 μM EDTA, 30 μM D,L-S-β-hydroxybutyryl-N-acetyl cysteamine and enzyme in a total volume of 1.5 ml. The rate of crotonyl thioester formation was measured at 263 mμ (46); *crotonyl thioester reduction*: cuvettes (d = 0.5 cm) contained 100 μM K-phosphate, pH 7.5, 10 μM EDTA, 0.5 μM DPNH or TPNH, 10 μM S-crotonyl-N-acetyl cysteamine and enzyme in a total volume of 1.0 ml. The oxidation of DPNH or TPNH was measured at 340 mμ against the control cuvette without substrate; *$C^{14}O_2$-malonyl CoA exchange reaction*: 50 μM Tris-buffer, pH 7.5, 5 μM EDTA, 5 μM cysteine, 0.1 μM malonyl CoA, 0.02 μM decanoyl CoA, 0.03 μM CoA and 1.2 μM $KHC^{14}O_3$ (1 μC/μM) in a total volume of 0.5 ml were incubated for 15 min at 30 C. The reaction was stopped by the addition of 0.5 ml 5 N acetic acid and the radioactivity measured.

even further is, possibly, a result of the summation of nonspecific damage, leading to the disruption of the organization of the total enzyme.

This idea leads to the most unusual aspect of fatty acid synthetase. That is, while purification data and physical properties, to be described, would lead us to envision a single enzymatic unit, we know that the synthetase catalyzes an orderly and progressive sequence of reactions. The structural unit can be visualized as a combination of six different enzymes arranged around a functional sulfhydryl group (Fig. 12), which firmly binds the intermediate stages in fatty acid synthesis in close proximity to the active sites of the component enzymes (8). In the picture we have drawn, the prosthetic group is fixed to a separate core, which, in itself, has no catalytic activity. Another possibility would be that the prosthetic group is firmly attached to one of the participating enzymes, possibly the one involved in the condensation.

The formulation of such a structure could explain: the multiplicity of enzyme activities observed to be catalyzed by the purified enzyme; the lack of free intermediates; and the physical properties of the enzyme.

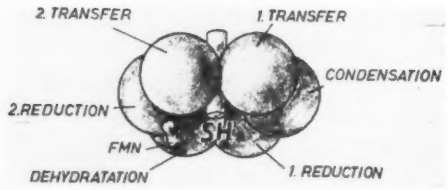


FIG. 12. Hypothetical structure of the "multienzyme" complex of fatty acid synthesis. The six enzyme units shown refer to the six reactions occurring in fatty acid synthesis:

1. transfer —*equation 2*
   condensation —*equation 3*
1. reduction —*equation 4*
   dehydratation —*equation 5*
2. reduction —*equation 6*
2. transfer —*equation 7*

The purified synthetase shows a single homogenous component in both the Tiselius electrophoresis apparatus and in the ultracentrifuge (unpublished E. Loren). A molecular weight of about 2 millions has been calculated from the sedimentation constant s = 29 [Svedberg]. In many respects the fatty acid system resembles the submicroscopic structure of the ketoacid oxydase complex (53). Such structural organizations can assure the correct combinations of substrates and cofactors in order to determine a specific structure of the product. There would seem to be a gradual transition from the relatively primitive enzyme complexes in this case to those involved in the more complicated reactions of protein synthesis in the microsomes and the multitude of functions in the mitochondria. As with the ketoacid oxidase complexes, it is expected that the component enzymatic activities of the synthetase can be dissociated from the functional unit. We have already partially purified a protein fraction of yeast having only the malonyl transferring activity (unpublished, A. Hagen).

A very interesting reaction is the last step of fatty acid synthesis catalyzed by this complex, the transfer of the synthesized saturated acid from the sulfhydryl group of the complex to coenzyme A (eq. 7). In contrast to the malonyl transfer, this reaction is specific for coenzyme A, and pantetheine or dephospho coenzyme A cannot replace coenzyme A. This enzymic activity is followed by measuring the rate of transfer of palmitic acid from palmityl CoA to radioactive C^{14}-CoA, as shown in eq. *18*, which is the sum of eq. *16* and *17*.

(*16*) palmityl CoA + enzyme $\rightleftharpoons$ CoA + palmityl-enzyme

(*17*) palmityl-enzyme + $\overset{*}{\text{C}}$oA $\rightleftharpoons$ enzyme + palmityl $\overset{*}{\text{C}}$oA

(*18*) palmityl CoA + $\overset{*}{\text{C}}$oA $\overset{\text{(enzyme)}}{\rightleftharpoons}$ CoA + palmityl $\overset{*}{\text{C}}$oA

Using various saturated acyl CoA derivatives, Eckhart Schweizer has studied the dependence of the rate of this acyl transfer on the chain length of the acid. The results are presented in Fig. 13. Although as yet fatty acids containing less than 14 carbons have not been studied, it is apparent that maximum activity is achieved with

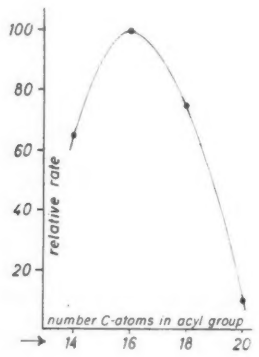


FIG. 13. Specificity of the enzyme component catalyzing the transfer of saturated fatty acids.

palmitic and stearic acid. This may explain why palmitic and stearic acids are the principal saturated fatty acids found in nature. Although at any given time all of the saturated fatty acids from C_4–C_{18} will be found in a mixture of synthetase molecules in comparable amounts, it is the specificity of this acyl transfer reaction that determines which fatty acids will be released from the complexes. It would be interesting to study this acyl transfer reaction in fatty acid synthesizing systems from other sources for comparison.

In summary, fatty acid synthesis as shown in Fig. 14, differs from the reversal of the degradative cycle (Fig. 1), in that, although we have the same type of acid intermediates, they are always enzyme bound. The fundamental difference is in the condensation reaction itself. The condensing unit is a thiolester derivative of malonate, rather than of acetate. As already brought out, the driving force is supplied by the concomitant decarboxylation of the malonyl derivative. Other differences of minor importance from the thermodynamic point of view, but very important from the mechanistic point of view, are listed in Table 3. These differences keep biosynthetic and degradative pathways separated.

For the living cell, there is a distinct safety factor in having the synthetic process separated from the degradative pathway. In the fatty acid system described

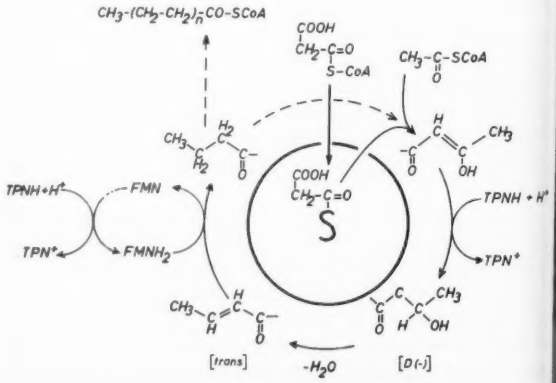


FIG. 14. Reaction scheme of fatty acid synthesis on the "multienzyme" complex.

TABLE 3. *Comparison of Compounds Involved in the Various Steps of Fatty Acid Degradation and Synthesis*

step	fatty acid degradation	fatty acid synthesis
SH - component	HSCoA	?
$C+C \rightleftharpoons \rangle C-C\langle$	Acetyl CoA	Malonyl CoA
$\rangle C=O \rightleftharpoons \rangle C(OH)H$	DPN, L(+)-HO-But.	TPN, D(-)-HO-But.
OH $-CH-CH_2- \rightleftharpoons -CH=CH-$	L(+)-HO-But.	D(-)-HO-But.
$-CH=CH- \rightleftharpoons -CH_2-CH_2-$	FAD	FMN

here, the price paid by the cell is one extra mole of ATP per C_2-unit condensed according to eq. *20* (Fig. 15).

I do not wish to overgeneralize, for it is certainly true that in some cases fatty acids are synthesized by reversal of the degradative pathway. Such is the case for the synthesis of butyric and capric acids by *Clostridium kluyveri* (54) and the liver fatty acid synthesizing system studied by Seubert et al. (10). However, for long chain fatty acid synthesis, the usual mechanism appears to involve the condensation of malonyl-CoA, a versatile intermediate in other reactions as well. We were particularly interested in the possibility of alternate reactions of malonyl CoA, because of the possibility that it was the biologically active condensing unit of the "polyacetate group" of natural substances (14), which includes phenols, pyrones, quinones, flavons, anthocyanins, depsides, and other aromatic compounds. In particular, it was Birch (55) who, since 1953, has drawn attention to the possibility that long chain poly-keto acids, formed by "head to tail" linkage of acetate units, could be the precursors to the structures of a large number of natural aromatic compounds. Such structures could be formed by the intramolecular cyclisation of various polyketoacid chains. Griseofulvin and eleutherinol are good examples of the projection of this concept (Fig. 16). The elucidation of the structure of tetracycline and macrolides by Woodward (56) further extended this idea.

Incorporation of C^{14} or O^{18} labeled acetate into various of these natural products showed the distribution of isotope anticipated from a polyacetate hypothesis [literature *cf.* (57)]. But the idea that polyketoacids are formed by repeated condensation of acetyl CoA did not appeal to me for theoretical reasons, namely the unfavorable equilibrium. This difficulty could be overcome if malonyl CoA were used (14). Recently, the first piece of experimental evidence supporting this proposal was obtained by M. Tada (57) in my laboratory. We investigated the biosynthesis of 6-methyl salicylic acid in Penicillium patulum, following the observation of Birch (58), and of Tanenbaum and Bassett (59), that acetate was the pre-

Reversal of fatty acid oxidation:

(*19*) $(n+1)$ Acetyl-SCoA + 2n (TPNH-DPNH) + 2n $H^+ \rightleftharpoons$ $CH_3-(CH_2-CH_2)_n-CO\text{-}SCoA$ + nHSCoA + 2n (TPN^+-DPN^+) + n H_2O

Malonyl-CoA-pathway:

(*20*) $(n+1)$ Acetyl-SCoA + 2n TPNH + 2n H^+ + n ATP $\xrightarrow{(CO_2)}$ $CH_3-(CH_2-CH_2)_n-CO\text{-}SCoA$ + n HSCoA + 2n TPN^+ + n ADP + nP

FIG. 15. Two pathways of fatty acid synthesis.

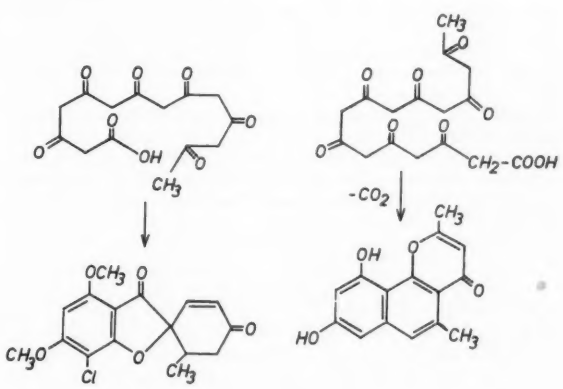


FIG. 16. Hypothetical precursors of Griseofulvin and Eleutherinol.

cursor of this compound. Fractionation of fungal extracts yielded a soluble preparation, which catalyzed the synthesis of 6-methyl salicylic acid from 1 mole of labeled acetyl CoA and 3 moles of malonyl CoA in the presence of TPNH. This process is illustrated by the eq. *21*:

(*21*) $CH_3-\overset{*}{C}O-SCoA + 3\ CH_2(COOH)-CO-SCoA + TPNH + H^+ \rightarrow$ 6-methyl salicylic acid + $3\ CO_2$ + $4\ HSCoA$ + TPN^+ + H_2O

Figure 17 illustrates the incorporation of radioactivity into 6-methyl salicylic acid by this enzyme preparation. For the separation of the aromatic acid from fatty acids, use was made of the formation of a complex of methyl salicylic acid with trivalent iron, using ferric chloride impregnated filter paper. After chromatography with benzene, the fatty acids were found close to the solvent front, whereas the 6-methyl salicylic acid remained at the origin. Subsequent development of the chromatogram with butanol saturated with water lead to the migration of the iron complex of methyl salicylic acid as a violet spot. The lower part of the figure shows a photograph of such a chromatogram, with the corresponding radioactive tracing above. As may be seen, the principal product is 6-methyl salicylic acid. Small amounts of fatty acids are also formed since the fatty

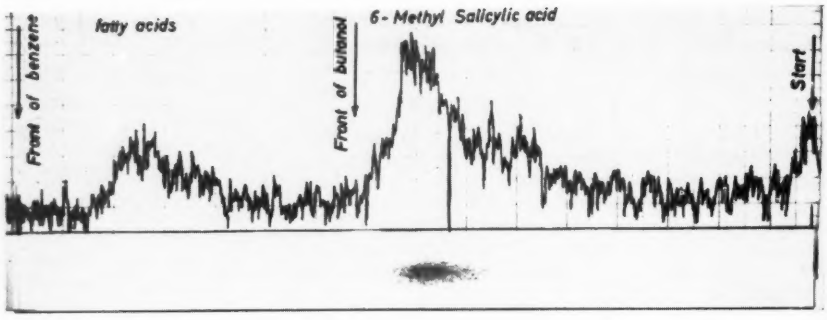


FIG. 17. Incorporation of C^{14}-acetyl CoA into 6-methyl salicylic acid. The incubation mixture contained 40 μM K-phosphate buffer, pH 6.85, 7 μM ethylenediamine tetraacetate, 0.1 μM TPN, 0.25 μM glucose-6-phosphate, 0.3 units glucose-6-phosphate dehydrogenase, 0.04 μM 1-C^{14}-acetyl CoA (150,000 cpm), 0.12 μM malonyl CoA, 0.76 mg enzyme fraction from Penicillium patulum and 0.06 μM 6-methyl salicylic acid as carrier. Total volume: 0.7 ml. After 2 hr incubation at 37 C the enzymic reaction was stopped and the thioester hydrolyzed by the addition of 0.08 ml 2 N KOH. The acidified mixture to which was added again carrier methyl salicylic acid (0.3 μM) was extracted with ether. The ether soluble material was chromatographed on Whatman No. 1 paper impregnated with 2% $FeCl_3$-solution as described in the text.

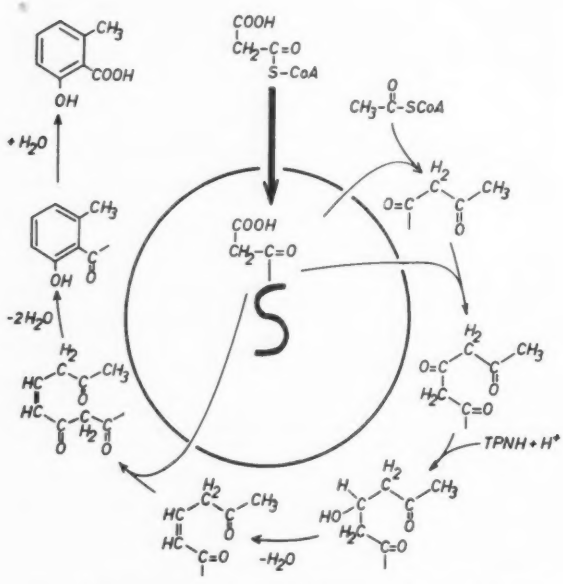


FIG. 18. Hypothetical reaction scheme of 6-methyl salicylic acid synthesis on a "multienzyme" complex.

acid synthetase has not been completely removed by the purification procedure.

It would seem that the enzyme system catalyzing the formation of aromatic structures resembles the enzyme complex encountered in the studies of fatty acid synthesis (Fig. 18). Similarly, it would seem that the intermediate ketoacids are bound to the complex by way of a thioester linkage. The observation that the synthesis is inhibited by iodoacetamide (57) would support this concept.

Such synthetase systems seem to represent a kind of template, on which the building units are assembled piece by piece, the product being liberated only after the process is completed. We then conclude that the specific arrangement of a number of common enzyme activities within the complex determines the direction of synthesis, that is, whether fatty acids, aromatic compounds, or macrolide structures are formed from malonyl CoA and TPNH in the presence of a suitable "primer," such as acetyl CoA and the same component enzymes.

The implications of this concept of a concerted enzyme action are profound and would appear to add a new dimension to enzymology, which raises many interesting and challenging problems. Certainly, among these, the question of the nature of the integral structure of the complex, the forces which are responsible for this specific structure, and the way in which the structure determines the product formed are of great importance. Equally important is the biochemical genetic mechanism whereby the information required for the precise structure of the enzyme complex can be transmitted from the parent cell to its progeny.

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Synthesis and metabolism of polyunsaturated acids

J. F. MEAD

University of California Medical Center, University of California, Berkeley, California

THE PRECEDING PAPERS IN THIS SYMPOSIUM have presented a very lucid description of the biosynthesis and metabolism of the saturated and monosaturated acids. Many of the reactions discussed here have applied equally to the polyunsaturated acids. However, from the initial investigations of these substances, it could be surmised that in many respects, they are quite different.

Although they are available as energy sources, it is evident that their most important functions stem from their participation in the structures of cellular membranes, terminal oxidation enzymes, and certain classes of blood lipoproteins. The original nutritional findings of Burr and his co-workers (1), indicated that these, unlike all other known fatty acids, could not be synthesized by the animal organism. This was confirmed by the tracer studies of Bernhard and Schoenheimer (2), and only recently has it been possible to show that under certain conditions, representatives of this class of compounds can be totally synthesized by animals.

Dietary Acid	Tissue Product
diencic (linoleic)	→tetraenoic
triencic (linolenic)	→pentaenoic + hexaenoic
none	→trienoic

FIG. 1. Conversions of polyunsaturated acids as revealed by alkaline isomerization.

These obvious differences led to considerable experimentation and speculation on the reactions of the polyunsaturated acids. However, until fairly recent years, the only information concerning their possible reactions was obtained indirectly with the techniques of alkaline isomerization. Holman and his co-workers (3, 4), and Reiser (5), obtained the following evidence: When rats on a fat-free diet were fed a dienoic acid (lnoleic), dienoic and tetraenoic acids were deposited in their tissues; when a trienoic acid (linolenic) was fed, pentaenoic and hexaenoic acids were deposited. On the other hand, if the fat-free diet was continued, these polyunsaturated acids decreased in the tissues and a trienoic acid increased (Fig. 1).

These findings were difficult to interpret and several "far-out" hypotheses were current, discussion of which might lead to some embarrassment. First hints as to the true nature of these reactions were seen in the tracer studies conducted in our laboratory and that of Klenk, and in the nutritional studies of Thomasson (6), who found that essentiality in a fatty acid appeared to depend on a specific structure of the terminal rather than the carboxyl end.

The reasons for these findings soon became apparent from the results of tracer studies. Since our work on the biogenesis of arachidonic acid has now been completed, I should like to use it as an illustration of the types of reactions to be considered. Briefly, the scheme of formation of arachidonic acid from linoleic is as in Fig. 2 (7–10):

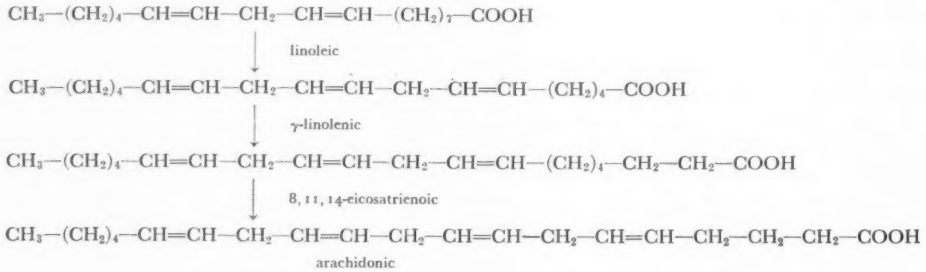

FIG. 2

This pathway was traced by a series of experiments involving the administration to rats of carbon-14-labeled acids, followed by location of the C^{14} in arachidonic acid. Acetate-1-C^{14} activity appeared only in the carboxy group of arachidonic acid. Linoleic-1-C^{14} acid activity appeared in carbon No. 1 as a result of its breakdown to acetate and in carbon No. 3 directly. Activity from γ-linolenic-1-C^{14} acid appeared almost exclusively in

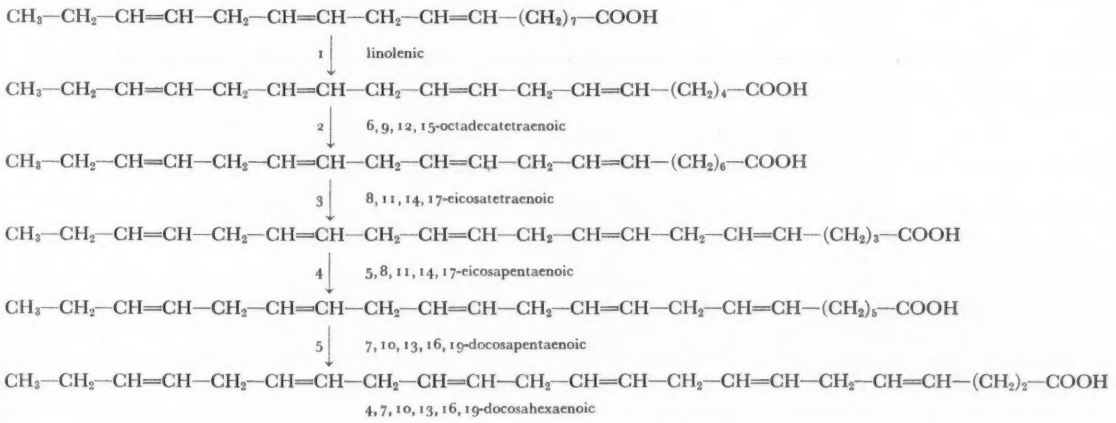

FIG. 3

carbon No. 3 of arachidonic acid, showing a very rapid conversion of the former into the latter. Finally, the activity from 8,11,14-eicosatrienoic-2,3-C^{14} acid appeared only in the corresponding carbons of arachidonic acid.

I do not mean to say that the pathway shown is the only possible route from linoleic to arachidonic acid, but it is certainly the major one and serves to illustrate the types of reactions contributing to the formation of the polyunsaturated acids. Starting from the existing double bonds of the precursor acid (in this case, linoleic), additional double bonds are introduced in the 1:4 relationship toward the carboxy group, until the next additional double bond would be in the $\alpha\beta$- or $\beta\gamma$-position. Chain-lengthening then permits the addition of one or more double bonds. In the case of the linoleate to arachidonic pathway, the process has almost stopped at arachidonic acid, although a 22-carbon acid in this series has been found.

Similar experiments with linolenic acid led to the conclusion that an almost identical pathway resulted in the ultimate formation of an eicosapentaenoic acid and docosapentaenoic and -hexaenoic acids (11, 12) (Fig. 3). Recently Klenk and Mohrhauer (13), in an elegant series of experiments, have demonstrated the reality of the previously hypothetical steps 2, 3 and 5 and, also, other conversions not on the proposed pathway. It seems evident that most of the possible reactions can occur under favorable circumstances.

These results indicated that the polyunsaturated fatty acids of the animal body could be built up from dietary unsaturated acids by the processes outlined for linoleic and linolenic acids. However, evidence soon appeared which indicated two different families of polyunsaturated acids. In an investigation of the nature of the trienoic acid which accumulates in the tissues of fat-deficient animals (3, 14), it was found that it was largely 5,8,11-eicosatrienoic, a dihydroarachidonic acid:

$$CH_3—(CH_2)_7—CH=CH—CH_2—CH=CH—CH_2—CH=CH—(CH_2)_3—COOH$$

Comparison of the activity incorporated into this acid from injected acetate-1-C^{14} with that of oleic acid isolated from the same rats revealed that the former acid was derived from the latter by a pathway similar to those previously shown (15). See Fig. 4.

The intermediate compounds in this pathway have also been found in fat-deficient rats.

In the same experiment, it was found that the eico-

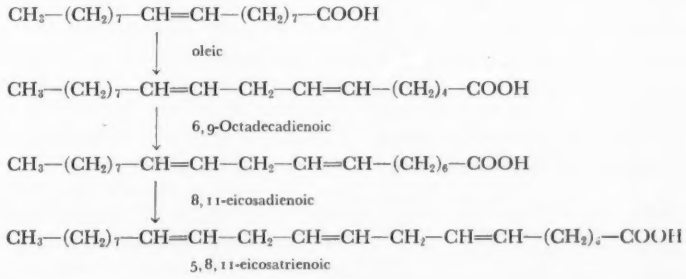

FIG. 4

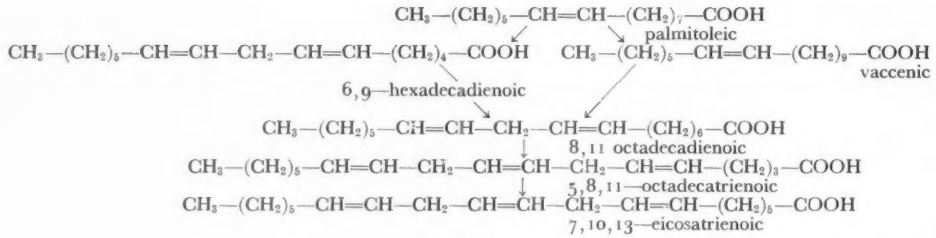

FIG. 5

satrienoic acid in fat-deficient rats also contained about 7% of the 7,10,13-isomer, which must have been derived from palmitoleic acid, a major fatty acid of fat-deficient animals. Other members of this family have also been found and a scheme of transformation can be formulated as in Fig. 5.

It is interesting that the ratio of the 7,10,13-eicosatrienoic acid to the 5,8,11-isomer is of the same order of magnitude as is the ratio of palmitoleic to oleic acid, indicating little selectivity by the enzymes for these particular isomers.

We may thus conclude that there are four families of polyunsaturated acids in the mammalian body, two derived from the dietary acids (linoleic and linolenic), and two derived from the monounsaturated acids (oleic and palmitoleic), which are themselves synthesized from acetate. These families are recognizable by the distance between the terminal methyl and the last double bond as in Fig. 6.

These families, which include all the presently known fatty acids commonly found in mammalian tissues, are not interconvertible, nor are the paths of formation of the higher members reversible, probably because of the hydrogenation steps.

It is interesting that in fish, in which the polyunsaturated acids generally make up a considerably larger percentage of the total, the 18-, 20-, and 22-carbon acids appear to be members of the same four families (16, 17). The 16-carbon acids, however, are quite different, and are either derived from the diet or formed in reactions unknown at present.

The enzyme systems responsible for the formation and interconversions of the polyunsaturated fatty acids have not been isolated and, indeed, their isolation may be a formidable task. Not only are the substrates and products easily destroyed under the conditions necessary for carrying out the reactions, but the actual types of enzymes sought are not clear. At present, it seems logical that three enzyme systems are operative—a polydehydrogenase which dehydrogenates mono- or polyenoic acids in positions in the 1:4 relationship from the existing double bonds toward the carboxy group; an acyl transferase, which adds 2 carbons when chain-lengthening is necessary; and a system which disposes of unsaturated acids with double bonds $\alpha\beta$ or $\beta\gamma$ to the carboxy group. The latter two enzyme systems have probably already been described. All three may have to be present before typical in vivo products can be produced in vitro.

$CH_3—CH_2—CH=CH—$ linolenic family
$CH_3(CH_2)_4—CH=CH—$ linoleic family
$CH_3—(CH_2)_5—CH=CH—$ palmitoleic family
$CH_3—(CH_2)_7—CH=CH—$ oleic family

FIG. 6

A further intriguing property of these enzymes relates to the temperature coefficient of their action. It has been known for many years that the polyunsaturated acids increase more rapidly in tissues kept at lower temperatures. This has recently been put on a clearer basis by the work of Kayama and Reiser (unpublished) in fish maintained at 10° or 25°. It seems unlikely that the reactions in question are more rapid at lower temperatures, so some other explanation must be advanced, such as greater inhibition of competing reactions at the lower temperature, or a lower rate of denaturation of the dehydrogenase.

In closing, I should like to say a few words concerning the function of the polyunsaturated acids in the mammalian body. Probably their physiological properties are largely dependent on their shape and consequent physical properties. Their low melting points and the attractive properties of their unsaturated centers permit them to act in the transport of many molecules, such as the cholesterol esters, in the essentially aqueous media of the body. Their shapes must also be responsible for their importance in the phospholipids of cellular membranes, and this property and, possibly, the lability of their allylic hydrogens, may be important in their function in the terminal oxidation enzymes. A logical question stemming from this discussion concerns the reasons for the importance of the linoleic family as essential fatty acids as compared with the other very similar polyunsaturated acids.

Here, again, the answer may be found in the shape of the principle end product of the family—in this case, arachidonic acid. It is possible that the important structural functions of arachidonic acid can be carried out only by an eicosatetraenoic acid, and that the principle end products of the other families simply do not quite fit the requirements.

As is always the case in research, I am afraid that as the metabolism of the polyunsaturated acids has become clearer, more questions previously unforeseen, have arisen. I presume that these, too, will yield to the efforts of the researcher within the next few years.

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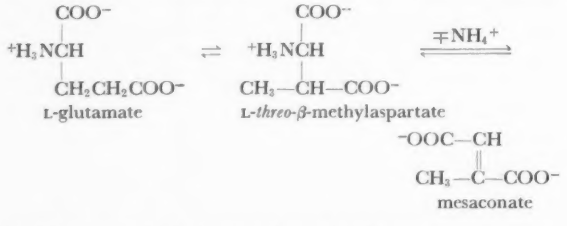
Structure and function of cobamide coenzymes

H. A. BARKER

Department of Biochemistry, University of California, Berkeley, California

WHILE "MECHANISM OF ACTION OF COENZYMES" is the topic of this Symposium, relatively little information is available concerning the mechanism of the cobamide or B_{12} coenzymes. They were first recognized less than 3 years ago. Consequently, most of the investigations of these compounds have been devoted to more elementary problems, such as the development of methods of assay and isolation, determination of properties and structure, exploration of natural occurrence, and participation in additional enzymatic reactions. The few enzymatic systems in which the coenzymes function have not been purified to a high degree. As a result, it has generally not been possible to approach the problem of the mechanism of cobamide coenzyme action under ideal conditions, likely to yield clear-cut and readily interpretable data. Nevertheless, some pertinent information has been obtained and some ideas have been advanced which I shall attempt to summarize and discuss. However, most of my talk will be devoted to a summary of the present knowledge of B_{12} coenzymes, with special emphasis on recent investigations concerning their structure and functions.

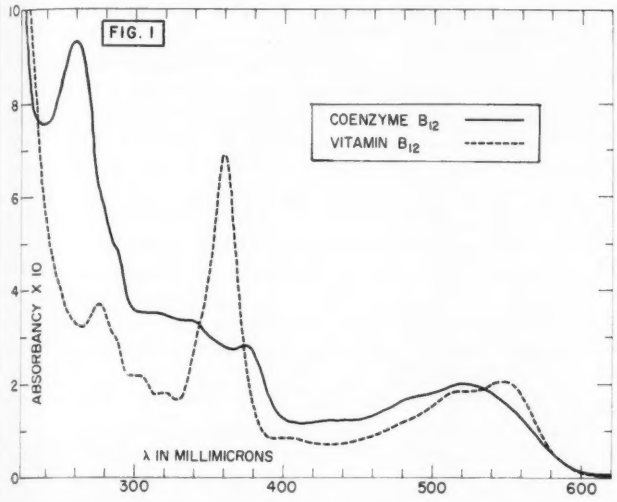
Coenzymes of the B_{12} group were first recognized during studies on the fermentation of glutamate by *Clostridium tetanomorphum* (3, 40). The initial enzymatic reaction



in this process is the conversion of L-glutamate to L-*threo*-β-methylaspartate, which I shall refer to as the glutamate isomerase reaction. This reaction is followed by a deamination of β-methylaspartate to mesaconate (4). The glutamate isomerase reaction was found to be dependent upon a heat-stable, light-sensitive cofactor, which could be removed from the enzyme system by adsorption on charcoal. This cofactor was eventually isolated and found to be a bright reddish-orange compound, which was identified as a coenzyme form of pseudovitamin B_{12} (3, 5, 41). Subsequently the analogous coenzyme form of vitamin B_{12} was isolated in larger amounts as a crystalline compound from propionic acid bacteria (6, 26).

Small amounts of six other coenzyme B_{12} analogues, containing variously substituted benzimidazoles or purines, in place of 5,6-dimethylbenzimidazole, have been isolated from *Propionibacterium arabinosum*, grown in the presence of the appropriate heterocyclic base (30, 37). And, finally, three corrinoid compounds that may be regarded as "incomplete coenzymes" have been either isolated from bacteria (Toohey, Smyth and Barker, unpublished results) or prepared by degradation of "complete" coenzymes (41). These compounds appear to be structurally similar to the complete coenzymes (Fig. 3), except that they lack the nucleotide base or the base and additional components of the nucleotide side chain. All of these "complete" and "incomplete" coenzymes have been tested for activity in the glutamate isomerase system. All of the complete coenzyme analogues are active. At saturating coenzyme concentrations, they all show approximately the same activity, although they differ markedly in affinity for the enzyme system. The incomplete coenzymes, which lack one or more of the components of the nucleotide, are entirely inactive. These results demonstrate that a complete nucleotide side chain is essential for coenzyme activity, but indicate that the nucleotide base is probably not the specific catalytic site.

Occurrence of the cobamide coenzymes and vitamins in bacteria and animal tissues has been investigated to a limited extent. All of the observations made so far indicate that the cobamide coenzymes are the predominant "natural forms" of the B_{12} group of compounds, at least in those organisms that contain relatively high levels of cobamides. The studies of Volcani et al. (38) and Perlman et al. (30) on bacteria have shown that a high percentage of the total vitamin B_{12} activity, frequently 80%



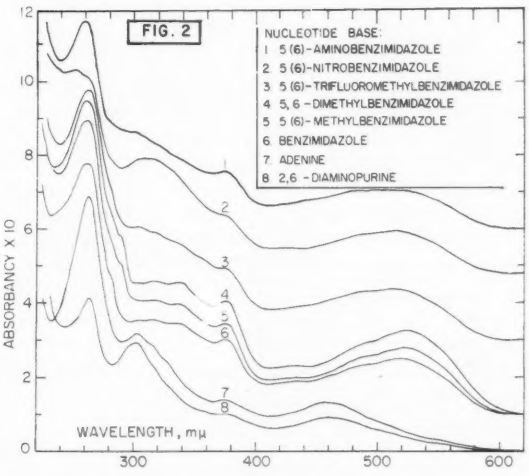


FIG. 1. Absorption spectra of coenzyme B_{12} and vitamin B_{12} (cyanocobalamin) in neutral solution. Coenzyme B_{12}, 2.69×10^{-5} M; vitamin B_{12}, 2.48×10^{-5} M.

FIG. 2. Absorption spectra of various cobamide coenzyme analogues in neutral solution (37). The analogues differ only with respect to the identity of the heterocyclic base in the nucleotide side chain.

or more, is in the coenzyme form. The vitamin forms, present in small amounts in some organisms, are usually the aquocobamides and cobinamide, which is vitamin B_{12} without its nucleotide side chain. The cyanocobamides, such as cyanocobalamin, apparently do not occur in nature in appreciable amounts. They were undoubtedly formed by the chemical degradation of the cobamide coenzymes.

Incidence of coenzyme B_{12} in the livers of several animals, including man, has been shown by Toohey and Barker (36), and has been confirmed for sheep liver by H. R. Marston and R. M. Smith (private communication) in Australia. The quantitative data show that about 50% of the vitamin B_{12} activity in alcohol extracts of liver can be isolated as coenzyme B_{12}. Since the isolation procedure is complicated, and the coenzyme is somewhat unstable, the true percentage of coenzyme may be considerably higher.

Properties of coenzymes B_{12} and its analogues are similar to those of vitamin B_{12}, but they differ sharply in some respects, so that the coenzyme and vitamin forms can be readily distinguished from each other. Absorption spectra of cyanocobalamin and coenzyme B_{12} in neutral solution are compared in Fig. 1. The spectra differ markedly with respect to the positions of the ultraviolet and visible absorbancy peaks. In general, the prominent peaks of the coenzyme are at shorter wave lengths than the corresponding peaks of the vitamin. The same sort of spectral difference between the coenzymes and vitamins is found with all of the cobamide analogues.

Various coenzyme analogues have generally similar absorption spectra; however, the benzimidazole-containing analogues can be readily distinguished from the purine-containing analogues by the position of the visible absorbancy maximum. This is shown in Fig 2 (37). The spectra of the six benzimidazole-containing analogues, labeled 1 to 6, have maxima at about 520 mμ whereas the spectra of the two purine-containing analogues, labeled 7 and 8, have maxima at about 460 mμ. This difference is especially significant, because it can be correlated with the well known preference of animals and certain microorganisms for the benzimidazole-containing cobamides. An investigation of the structural basis of the spectral differences between the purine- and benzimidazole-containing coenzymes has led to the conclusion that it is attributable to a much weaker binding of the purines to the cobalt atom (24). In fact, since the spectra of the purine-containing analogues are not appreciably altered by complete removal of the purine, the latter appears not to be effectively bound to the cobalt atom. The absence of a substantial bond between the purine and cobalt should permit the nucleotide side chain to move about freely, within the restrictions of its primary structure, and, thus, greatly modify the shape of the molecule. This, in turn, may account for the inability of animal systems to utilize the purine-containing vitamins and coenzymes.

Cobamide coenzymes, as a group, differ chemically from the vitamins by having an extra adenine and sugar moiety, probably present in the form of a nucleoside (21, 23, 41). The adenine and sugar are readily removed from the coenzyme by exposure to light or by treatment with alkaline cyanide. Photolysis of coenzyme B_{12} in the presence of oxygen gives an adenine nucleoside and aquocobalamin, whereas treatment with cyanide ion yields adenine, cyanocobalamin, and probably the cyanohydrin of the sugar, although this product has not yet been characterized. These reactions have been useful in establishing the structural relation between the coenzymes and vitamins.

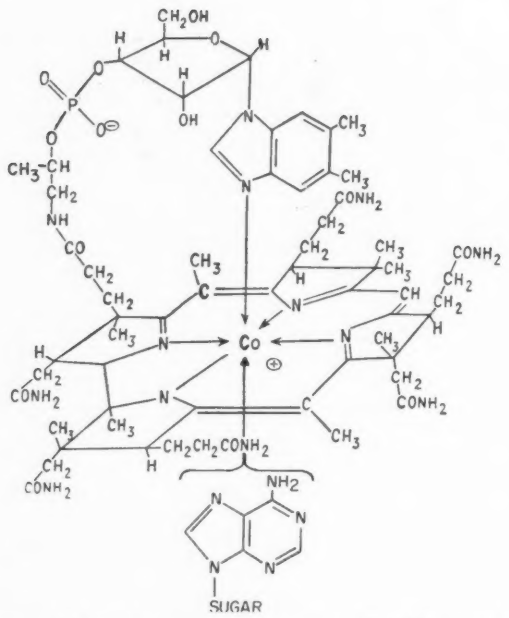


FIG. 3. Schematic structure of coenzyme B_{12}. The complete structure of the adenine nucleoside and its mode of attachment to the corrinoid ring system are not known.

The complete structure of coenzyme B_{12} is not yet known. However, Fig. 3 shows a partly schematic and hypothetical structure, which embodies some of the information now available. The figure represents coenzyme B_{12} as a derivative of vitamin B_{12}, in which the coordination position sometimes occupied by the cyanide ion is occupied by an adenine nucleoside, and in which the cobalt is in the divalent form. The adenine nucleoside is shown to be attached to the cobalt atom, because a bonding of adenine to cobalt is known to occur in other cobamides, and because the displacement of adenine by CN^- and of the nucleoside by light provides some support for this interpretation. However, the identity of the atom or moiety bonded to the cobalt atom is not yet established.[1]

The identity of the extra sugar in coenzyme B_{12} has been under investigation for some time, first by Wiessbach et al. (41), who demonstrated that it is not ribose or any one of the common sugars; then by Ladd et al. (23), who determined a number of its properties; and most recently by Hogenkamp and Barker (20), who obtained substantial evidence concerning its structure. Determination of the structure has progressed slowly, partly because we were inexperienced in this type of investigation, and partly because of the relatively small amounts of material available.

The sugar has generally been released by mild acid hydrolysis (0.1 N, 100°, 90 min) of coenzyme B_{12}, and then separated from other products by means of ion exchange resins. The sugar can be obtained free from ribose, which is also present in the coenzyme, because the ribosebenzimidazole bond is very stable to acid hydrolysis. The structure of the sugar has so far been investigated only by analytical and degradative procedures. The results indicate the sugar has the structure shown on the left in Fig. 4. It is actually a five-carbon aldehyde containing two hydroxyl groups and a terminal double bond which can be called D-*erythro*-2,3-dihydroxy-Δ4-pentenal (20). The terminal position of the double bond is established by the formation of formaldehyde in good yield by ozonolysis of the sugar.

The remaining dialdehyde can be reduced with sodium borohydride to a polyalcohol, which has been identified as erythritol. The configuration of the hydroxyl groups has been determined by converting the top four carbon atoms of the sugar to erythrose, as shown in Fig. 5. The aldehyde group of the sugar was first reduced with sodium borohydride and then the double bond was cleaved by ozonolysis. The resulting tetrose was identified by paper chromatography as erythrose. It was shown to have a positive optical rotation and therefore must be L-erythrose. Consequently, the original sugar, which has the aldehyde group on the opposite end, must have the D-*erythro* configuration. Further evidence for the proposed structure was obtained by the action of osmium tetroxide on the original sugar. This reagent hydroxylates the two carbons joined by the double bond with the formation of approximately equal amounts or ribose and lyxose, which were identified chromatographically. These and other lines of evidence support the proposed structure, which, however, should be regarded as tentative until confirmed by synthesis. It must also be emphasized that this compound is a product of mild acid hydrolysis of the coenzyme. It is possible, and even probable, that the original sugar in the coenzyme has been somewhat modified by the hydrolytic procedure. This possibility requires further investigation.

The problem of the valence of the cobalt in the cobamide coenzymes deserves some consideration, particularly in view of the fact that Eggerer et al. (14, 15) have postulated that the coenzyme cobalt functions as a reversible redox system. In vitamin B_{12}, the cobalt is known to exist in the trivalent state. This has been established by magnetic susceptibility measurements (39) and by X-ray spectroscopy (10). Reduction of vitamin B_{12} to vitamin B_{12r}, which contains divalent cobalt, can be effected only with powerful reducing agents, such as chromous salts or hydrogen and palladium (7, 13); the reduced form of the vitamin is relatively unstable and is rapidly reoxidized by oxygen to the trivalent state. In the B_{12} coenzymes, on the contrary, the stable form of the cobalt is almost certainly divalent. This was initially indicated by the striking differences in the spec-

[1] G. Lenhert and D. Hodgkin have obtained evidence by X-ray diffraction methods that the adenine nucleoside moiety present in coenzyme B_{12} has the structure of adenosine except that it lacks the 5′ hydroxyl group. The 5′ carbon of the sugar appears to be attached directly to cobalt. These results were reported at the Second European Symposium on Vitamin B_{12} and Intrinsic Factor held in Hamburg, Germany, August 2–5, 1961. They will be published in the proceedings of the Symposium and separately in *Nature*.

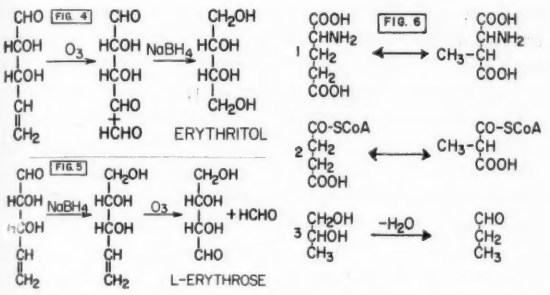


FIG. 4. Conversion of the sugar to formaldehyde and erythritol by ozonolysis followed by reduction with borohydride (20).

FIG. 5. Conversion of the sugar to formaldehyde and L-erythrose by borohydride reduction followed by ozonolysis (20).

FIG. 6. Reactions requiring cobamide coenzymes.

tra of the B_{12} vitamins and coenzymes and, more specifically, by the fact that the coenzymes, like the cyanocobamide vitamins, have a net zero charge in neutral solution, even though they lack the negatively charged cyanide group (41).

Further evidence for divalent cobalt in the coenzymes was recently obtained by Brady and Barker (11). They studied the influence of oxygen on the nature of the products formed by photolysis of coenzyme B_{12} in an extension of an earlier study by Pawelkiewicz et al. (29). In the presence of oxygen, the coenzyme is converted to aquocobalamin which contains trivalent cobalt. In the absence of oxygen, the coenzyme is quantitatively converted by light to vitamin B_{12r}, which is readily identified by its spectrum, which has a prominent absorbancy peak at 475 mμ. This compound is stable in the absence of oxygen, but is immediately oxidized to aquocobalamin on exposure to air. The latter compound can be recognized by its high absorbancy peak at 350 mμ. The quantitative formation of the reduced vitamin in anaerobic photolysis suggests that the coenzyme itself contains divalent cobalt. This evidence is not conclusive, because the possibility that the cobalt is reduced during photolysis cannot be excluded.

More conclusive evidence that the cobalt in the coenzyme is divalent was recently reported by Bernhauer et al. (8, 9) and by Nowicki and Pawelkiewicz (28). These investigators found that in solution coenzyme B_{12} and a structurally related compound, probably an incomplete cobamide coenzyme, are paramagnetic, the observed magnetic moment (1.3 to 1.63 Bohr magnetons) being approximately that expected for an octahedral cobaltous complex containing one unpaired electron. Under similar conditions, cyanocobalamin is diamagnetic. These results, and the other types of evidence I have mentioned, support the conclusion that the stable form of cobalt in coenzyme B_{12} is divalent. So far as I am aware, there is no evidence that the valency of the cobalt can be changed without destroying the activity of the coenzyme, although the possibility that this can occur under some conditions is obviously not excluded.

Let us now consider the functions of the cobamide coenzymes. The coenzymes are known to be required for three enzymatic reactions, all of which occur in some microorganisms, and one of which occurs in animals. These reactions are shown in Fig. 6.

The first is the glutamate isomerase reaction, which so far has been observed only in *C. tetanomorphum*. This reaction involves a reversible transfer of a two-carbon "glycine" moiety between carbon atoms 3 and 4 of glutamate (27). Since the lower three carbon atoms of glutamate and the associated atoms constitute a substituted propionate molecule, the reaction can be regarded as a transfer of a "glycine" moiety between the α and β carbon atoms of a propionate molecule, with a simultaneous transfer of hydrogen in the opposite direction. The reaction is apparently complex, since it is catalyzed by two separable protein fractions, neither of which is active by itself (Iodice, Weissbach, Smyth, and Barker, unpublished results). So far no intermediates have been detected, either as a result of direct accumulation, or by exchange reactions with isotope-labeled substrates. By the latter technique, the more obvious possible intermediates, such as free glycine, propionate, acrylate and α-ketoglutarate, have been excluded. For technical reasons, it has not yet been possible to determine whether the nitrogen of the amino group remains attached to the carbon during the isomerization reaction.

The second is the succinyl-methylmalonyl isomerase reaction, which occurs both in animals and bacteria (16, 18, 32–34). As is well known, this reaction is involved in the metabolism of propionate and it appears to be of special importance in ruminants which use propionate as a major energy source. Eggerer et al. (14, 15) have shown by tracer experiments that in this reaction the thioester carboxyl group moves between the α and β carbons of the propionate portion of the molecule. Consequently, this reaction is completely analogous to the glutamate isomerase reaction.

Bacterial and animal methylmalonyl isomerase systems differ with respect to their coenzyme specificities and, also, with respect to the strength of binding of the prosthetic group to the enzymes. The isomerase derived from *Propionibacterium shermanii* holds the coenzyme rather loosely, as Stadtman et al. (32) have shown. The coenzyme can be effectively removed by treating the enzyme preparations with charcoal. The resolved enzyme system can be reactivated with either the adenine-, benzimidazole- or 5,6-dimethylbenzimidazole-containing coenzyme analogue. The K_m values for the various analogues differ by only a factor of 10. Coenzyme B_{12} has the lowest K_m value of 2.4×10^{-8} M; this is about 1,000 times smaller than the K_m of this coenzyme in the glutamate isomerase system. The methylmalonyl isomerase system derived from sheep kidney binds its prosthetic group very strongly, as Lengyel et al. (25) have shown. In this sytem the prosthetic group cannot be removed by charcoal treatment, but only by treating the enzyme with acid ammonium sulfate. The resolved enzyme system is reactivated by coenzyme B_{12} and its benzimidazole-containing analogue but not by the

adenine-containing analogue. The K_m of coenzyme B_{12} with the resolved enzyme is about the same, 2.1×10^{-8} M, as that for the bacterial isomerase.

These results indicate that the coenzyme in the animal isomerase system is bound much more strongly before than after resolution with acid ammonium sulfate. A special type of binding of the prosthetic group to the native enzyme is further suggested by the observation that the enzyme cannot be inactivated by exposure to light under conditions that rapidly inactivate solutions of the B_{12} coenzymes. In this connection it should be noted that the stability of coenzyme B_{12} toward light is greatly influenced by its physical state. In aqueous solution the coenzyme is inactivated by light about 900 times more rapidly than in the crystalline state (Hogenkamp and Barker, unpublished results). Consequently, it is not surprising that protein-bound coenzyme is sometimes more stable toward light and certain other reagents than is the free coenzyme.

One of the most significant developments during the past year was the discovery by Abeles and Lee (1, 2) of the third B_{12} coenzyme-dependent reaction shown in Fig. 6. This reaction, which is catalyzed by extracts of *Aerobacter aerogenes*, is quite different from the two isomerase reactions. It is an intramolecular oxidation-reduction accompanied by a removal of the elements of water. The substrate is a glycol, which is converted to the corresponding 2-deoxyaldehyde. The third reaction in Fig. 6 shows the conversion of 1,2-propanediol, the best substrate, to propionaldehyde. The enzyme system can also convert ethyleneglycol to acetaldehyde. Both of these reactions obviously involve the transfer of a hydrogen atom from carbon 1 to carbon 2. Brownstein and Abeles (12) have investigated the mechanism of the hydrogen transfer by carrying out the reaction in the presence of D_2O and determining the deuterium content of the product, isolated as the crystalline hydrozone. They found no significant incorporation of deuterium into the aldehyde which could be attributed to the enzymatic reaction. This result excludes reaction mechanisms involving the intermediate formation of a free proton. It supports the view that the hydrogen is moved as a hydride ion or a hydrogen atom, although Brownstein and Abeles recognized the possibility that the hydrogen could be moved as a proton, provided the active site on the enzyme or coenzyme were so constituted as to exclude exchange with the solvent during the transfer.

In my laboratory, Dr. Iodice (unpublished data) has done similar experiments with the glutamate isomerase reaction. In this reaction, also, one hydrogen must be transferred during the conversion of the methylene group of glutamate to the methyl group of β-methylaspartate or mesaconate, which should be equivalent. For technical reasons, mesaconate, rather than β-methylaspartate, has been isolated and examined for isotope incorporation following the enzymatic isomerization of glutamate in the presence of labeled water. These experiments were done initially with tritium oxide in the solvent, and the results showed an extremely small incorporation of tritium, equivalent to less than 0.01 of an atom of tritium per mole of mesaconate. However, in view of the well known isotope selection against tritium in some reactions, Dr. Iodice repeated the experiments using 100% D_2O as the solvent. Under these circumstances the mesaconate formed from glutamate contained only about 0.10 atom of deuterium per mole of mesaconate This small incorporation of deuterium is probably the result of a side reaction, the reversible oxidation of glutamate to α-ketoglutarate, which by an enolic rearrangement slowly takes up deuterium from the solvent. Various control experiments have eliminated the possibility that a significant amount of deuterium can have been lost during the isolation and purification of the mesaconate. We must conclude that a free proton is not an intermediate in either the glutamate isomerase reaction or the glycol-deoxyaldehyde reaction.

The results of these deuterium exchange experiments appear to be inconsistent with the free radical mechanism of cobamide coenzyme action proposed by Eggerer et al. (14, 15) for the methylmalonyl and glutamate isomerase reactions. Their hypothesis assumes the formation of a free radical by removal of a hydrogen atom from the substrate; the hydrogen atom reacts with trivalent cobalt of the coenzyme to form a proton and divalent cobalt. Following a rearrangement of the free radical, the proton is reduced by the divalent cobalt to a hydrogen atom, which then returns to the substrate molecule to complete the isomerization. This hypothesis involves the formation of a proton as an intermediate and necessitates the incorporation of at least one atom of hydrogen or deuterium from the solvent. Although the possible incorporation of solvent deuterium into the products of the methylmalonyl isomerase reaction has not yet been investigated, it now seems unlikely that this will occur as an enzymatic process.

The limited information so far available suggests that the B_{12} coenzymes function by catalyzing the transfer of hydrogen, possibly in the form of a hydride ion, between adjacent carbon atoms of the substrate, but other explanations cannot be excluded. The active site on the coenzyme is not yet established. The cobalt atom has been suggested as a possible reaction site, but it must be noted that the cobalt atom appears to be so deeply imbedded in the coenzyme structure that direct contact of the substrate with the cobalt is unlikely. Furthermore, a direct reaction of cobalt with a hydrogen atom or hydride ion would probably result in the formation of a proton, which is inconsistent with the results of the deuterium exchange experiments. It is more likely that contact between the coenzyme and the substrate involves some peripheral part of the coenzyme molecule. Smith (31a) suggested a few years ago that one of the amide groups may be the site of catalytic activity.

However, since a hydrogen transfer function of the coenzyme is now indicated, it seems more probable that some peripheral part of the coenzyme more closely associated with the conjugated double bond system may

be involved. Further speculation concerning the active site of the coenzyme does not seem useful until the structure of the coenzyme has been determined in more detail.

Finally, may I point out that the B_{12} coenzymes which I have considered are only one of several types of catalytically active corrinoid compounds presently emerging. Dr. Rabinowitz has discovered (31), and will soon discuss, the extraordinary coenzymatic activity of cobinamide and other corrinoid compounds formerly regarded as incomplete B_{12} vitamins. Investigations of the biosynthesis of methionine (17, 19, 22, 31, 35) have established the existence of protein-bound corrinoid compounds that may turn out to be structurally somewhat different from the known corrinoid vitamins and coenzymes. It seems probable that soon there will be a family of corrinoid coenzymes comparable to that of the folic acid series.

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Role of vitamin B_{12} derivatives in reactions of pyruvate

JESSE C. RABINOWITZ AND ESTHER H. ALLEN[1]
Department of Biochemistry, University of California, Berkeley, California

OVER THE PAST FEW YEARS we have been investigating the fermentation of purines by two closely related clostridia, which were originally studied by Barker and Beck (2). Extracts of both organisms degrade xanthine and several other purines to CO_2 ammonia, and formiminoglycine (Fig. 1). Formiminoglycine is converted to glycine and 10-formyl-THF[2] by extracts of both organisms, when supplemented with THF (Fig. 2), in a series of five enzymic reactions (10). The subsequent metabolism of these products differs in the two organisms, and yields the products characteristic of each fermentation. 10-Formyl-THF may be acted on by the enzyme THF formylase in the presence of ADP and inorganic orthophosphate to yield formic acid and ATP, and regenerate the tetrahydrofolic acid. The products, formic acid and glycine, are characteristic of the fermentation of purines by *C. cylindrosporum* (12). However, in *Clostridium acidi-urici*, relatively small amounts of formate accumulate, and no glycine is detectible. Instead, acetate and CO_2 appear as end products (2).

The formation of these compounds is most readily explained by the alternate series of reactions shown in Fig. 2, in which glycine and 10-formyl-THF are converted to serine by serine aldolase. Serine is then acted on by serine deaminase to form pyruvate, which is finally oxidized by a pyruvic oxidase to acetate and CO_2. Indirect evidence exists for the occurrence in these organisms of all the enzymes required for these transformations (3, 5, 14, 15), and the results of fermentation experiments using formiminoglycine-C^{14} are consistent with the postulated mechanism (13). We became interested in the final reaction of this sequence—the oxidation of pyruvic acid to acetate and CO_2—since bacterial enzymes of this type have generally not been obtained in highly purified form, and since this reaction appears to be the main energy-yielding reaction in the fermentation of purines by *C. acidi-urici*.

An assay was used in which the exchange of $C^{14}O_2$ into pyruvic acid was determined. The assay system is shown in Table 1. Activity is expressed in terms of counts per min per mg of 2,4-dinitrophenylhydrazone isolated. It was shown that the $C^{14}O_2$ was incorporated exclusively into the carboxyl group of the pyruvic acid (11). The results obtained using this assay system are shown in Fig. 3, and demonstrate that with relatively low concentrations of bacterial extracts, the radioactivity incorporated in the phenylhydrazone, is proportional to the amount of enzyme added. The relative extent of exchange was determined (Fig. 4), and it was found that with 0.3 mg of protein, roughly 20% exchange was observed in a 30-min period. Although greater amounts of exchange were obtained after longer incubation periods, the reaction is no longer linear. The enzyme has been partially purified by conventional techniques using this assay, and a preparation purified approximately tenfold over the starting material has been obtained. This partially purified preparation, in contrast to the initial extracts, is very stable and it has been used for all the studies which will be reported.

The specificity of the reaction with respect to the various α-keto acids is shown in Table 2. The exchange was entirely specific for pyruvic acid. The system showed relatively low exchange when formic acid was substituted for CO_2. The exchange with acetate-C^{14} was barely detectible, and acetyl-coenzyme A also failed to exchange to any significant extent. The nature of the two-carbon fragment formed from pyruvate is still not known.

When the bacterial extracts are treated with Dowex-1 chloride, the exchange reaction is dependent on the addition of several cofactors shown in Table 3. The cofactors required are cobinamide or some other vitamin B_{12} derivative; coenzyme A; and a sulfhydryl compound, such as 2-mercaptoethanol. The activity obtained with these three cofactors in the presence of a phosphate buffer, pyruvate, and bicarbonate, was somewhat better than that obtained using a boiled extract of the organism as a source of cofactors. Other cofactors tested, including thiamine pyrophosphate, lipoic acid, DPN, TPN, FAD, and ATP, had no effect on the reaction, nor was the reaction inhibited by EDTA. Avidin,

[1] Postdoctoral Research Fellow, Division of General Medical Sciences, Public Health Service.

[2] The abbreviations used are: THF, tetrahydrofolic acid (the various one-carbon derivatives of this compound are indicated by the prefix and the number refers to the position of substitution on the pteridine ring); EDTA, ethylenediaminetetraacetic acid; MET, 2-mercaptoethanol.

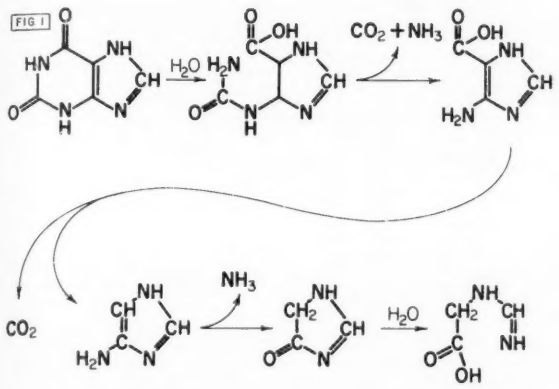


FIG. 1. Intermediates in the degradation of unsupplemented extracts of *Clostridium cylindrosporum* and *Clostridium acidi-urici*.

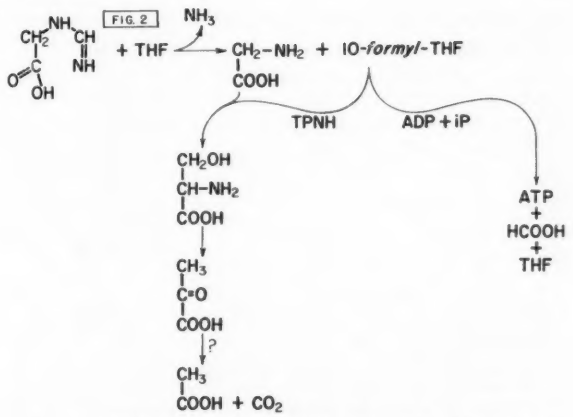


FIG. 2. Metabolism of formiminoglycine by *Clostridium cylindrosporum* and *Clostridium acidi-urici*.

in concentrations approximately 10 times higher than those used to inhibit other avidin-sensitive enzyme systems, had no effect on the exchange reaction. This CO_2-pyruvate exchange reaction, therefore, differs from the avidin-sensitive reaction of *C. kluyveri* recently reported by Shuster and Lynen (17).

The dependence of the exchange reaction on the concentration of 2-mercaptoethanol is shown in Fig. 5. Relatively large amounts are required, and 200 μmoles of 2-mercaptoethanol are included per ml of reaction mixture in the routine assay. Mercaptoethylamine, mercaptoacetic acid, and cysteine are as effective as mercaptoethanol. However, sodium sulfide, ethylmercaptan, and sodium ascorbate are completely inactive in satisfying this requirement.

The specificity of the corrin requirement was determined. I should like to review the nomenclature and structures of this series of compounds (19). The structure of the cobamide coenzyme discovered by Barker and his co-workers (1) is shown in Fig. 6. The removal of the purine-sugar moiety yields vitamin B_{12} or the cobamides. A series of cobamides have been obtained, which differ from one another either in the nature of the inorganic ligands, which satisfy the sixth coordinate valence of cobalt, and members of the series may contain cyano- or aquo- groups; or in the nature of the base of the nucleotide moiety. The dimethylbenzimidazole shown here may be replaced by adenine. Derivatives of the

TABLE 1. *Assay for CO_2-Pyruvate Exchange Reaction*

	μmoles/ml
Phosphate buffer, pH 7	225
Na pyruvate	50
Na bicarbonate-C^{14} (176,000 cpm)	10
Cofactors	
Enzyme	

Incubated at 37° for 30 min. The reaction was stopped by the addition of 0.1 ml of 20% perchloric acid per ml. The protein was removed and 2,4-dinitrophenylhydrazine was added. The 2,4-dinitrophenylhydrazone was purified, plated by filtration, and its radioactivity was determined.

cobamides have been prepared by substituting methylamine or aniline for one of the amide groups on the propionyl sidechain (18). These compounds show relatively high antivitamin activity. Further degradation of the molecule by removal of the nucleotide portion leads to a series of compounds, which are referred to as cobyrinic acid derivatives. The structure obtained on removal of the nucleotide from any form of vitamin B_{12} is cobinamide or Factor B. Removal of the propanolamine sidechain yields the cobyrinic acid hexamide or Factor V_{1a} of Bernhauer and associates (4), and finally the so-called hexacarboxylic acid is obtained by alkaline hydrolysis of the vitamin and removal of all the amide groups from the sidechains and cyclization of propanolamine moiety (19).

The corrin derivatives listed in Table 4 are all active in stimulating the CO_2-pyruvate exchange reaction. The relative activities are indicated here by the amounts required for stimulation of the exchange activity to half-maximum amount and are expressed as K_m in moles per liter. The maximum reaction velocity observed with all of these compounds was approximately the same. The most active compounds tested were the most degraded forms of the coenzyme, that is the hexacarboxylic acid, the cobyrinic acid hexamide, and cobinamide. These compounds had K_m values between 1 and 2×10^{-9} M, and near maximal activity is obtained with as little as 1.0×10^{-6} μmoles/ml of these substances. The cobamide vitamins were the next most active series of compounds. The methylamide and anilide of cobalamine, which are active antagonists of bacterial growth, were quite active as coenzymes in the exchange reaction. The least active of the compounds tested were the cobamide coenzymes described by Barker. The activities of the compounds reported here were obtained using precautions to protect them from light inactivation. It is not altogether certain that the activity obtained with these compounds was, in fact, due to the coenzyme itself or to very small amounts of contaminating cobyrinic acid derivatives. The activity observed with the cobamide coenzymes

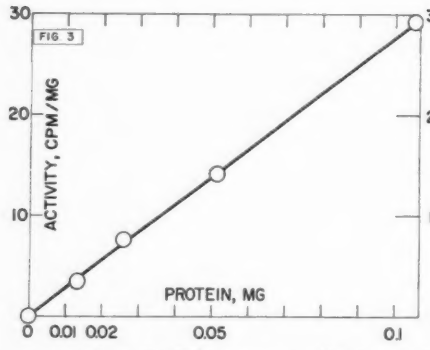


FIG. 3. Assay for CO_2-pyruvate exchange reaction. The activity is expressed as counts per minute per mg of 2,4-dinotrophenylhydrazone. The conditions of the assay are as described in Table 1, with 0.01 μmole of CoA and 2×10^{-5} μmoles of cobinamide added.

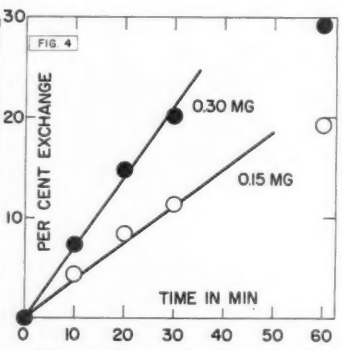
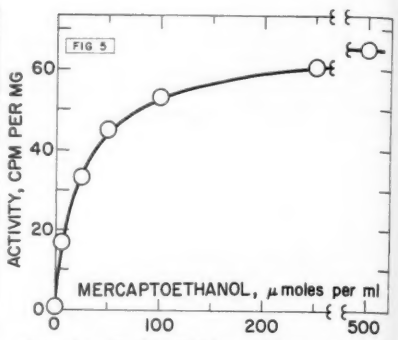


FIG. 4. Per cent exchange in CO_2-pyruvate exchange reaction.



Assay conditions are those described in Table 1 with 0.15 or 0.30 mg of enzyme protein, 0.01 μmole of CoA and 2×10^{-5} μmoles of cobinamide.

FIG. 5. Sulfhydryl requirement for exchange reaction.

TABLE 2. *Specificity of Exchange Reaction*

α-Keto Acid	Labeled Compound	Relative Exchange
Pyruvate	$C^{14}O_2$	100
α-Keto-butyrate	$C^{14}O_2$	0
α-Keto-valerate	$C^{14}O_2$	0
α-Keto-isovalerate	$C^{14}O_2$	0
α-Keto-glutarate	$C^{14}O_2$	0
Oxaloacetate	$C^{14}O_2$	0
Pyruvate	$HC^{14}OOH$	3.0
Pyruvate	$CH_3—C^{14}OOH$	0.5
Pyruvate	$CH_3—C^{14}O$-SCoA	0.5

The assay conditions used are described in Table 1, with 0.03 mg of enzyme protein, and the α-keto acid and C^{14}-containing compound indicated in place of pyruvate and bicarbonate-C^{14}. The activity is expressed as cpm per mg of 2,4-dinitrophenylhydrazine isolated.

TABLE 3. *Cofactor Requirements for Exchange Reaction*

Assay System	Exchange Activity cmp/mg Hydrazone
Complete	80.7
Minus cobinamide	2.2
Minus coenzyme A	2.1
Minus 2-mercaptoethanol	0.54
Minus cobinamide and CoA plus boiled extract	56.0

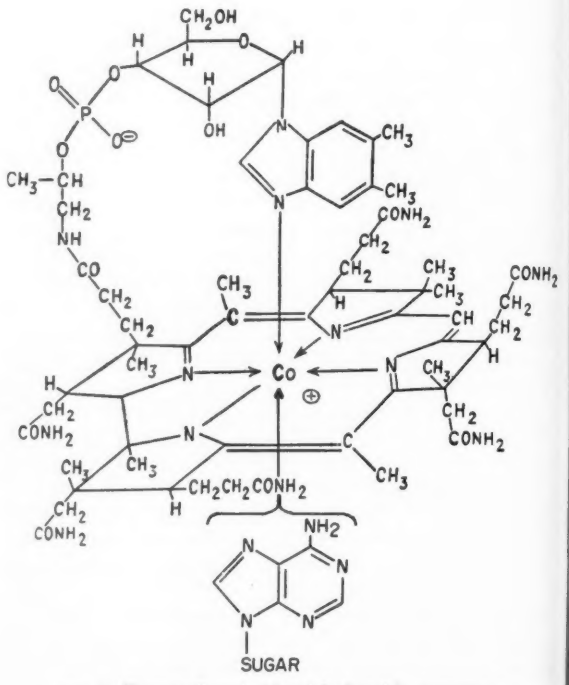


FIG. 6. Dimethylbenzimidazolylcobamide coenzyme.

could be explained if they were contaminated with only 2,000ths of a per cent of cobyrinic acid derivatives. It is, therefore, possible that the cobamide coenzymes are completely inactive in the system. Finally, it was noted that the exchange system is stimulated by cobaltous chloride. Although the K_m of cobaltous chloride is only 1×10^{-3} M, approximately one million times more cobalt chloride is required for coenzyme activity than any one of the cobyrinic acid derivatives. This large difference reflects the extraordinary activity of the cobyrinic acid derivatives in this system.

A number of other compounds which were tested for coenzyme activity are listed in Table 5. The cobaltous porphyrin complexes (6) tested had no coenzyme activity, nor did they inhibit the action of cobinamide. Various complexes of cobaltic ion were tested. Most of these contained organic ligands. None of the compounds tested showed any coenzyme activity, and a number of those tested indicated here were fairly active inhibitors of the exchange system when tested in the presence of cobinamide. The two other transition metals in group 8, which includes cobalt, were tested, and both showed some coenzyme activity. It, therefore, appears that the corrin requirement for the pyruvate-CO_2 exchange reac-

TABLE 4. *Specificity of Corrin Requirement for CO_2-Pyruvate Exchange*

	K_m[M]
Cobyrinic acid derivatives	
Hexacarboxylic acid*	1.5×10^{-9}
Cobyrinic acid hexamide (factor V_{1a})†	2.2×10^{-9}
Cobinamide (factor B)‡	1.5×10^{-9}
Cobamides (vitamins)	
Adeninylcobamide (pseudo vitamin B_{12})	3×10^{-8}
Aquocobalamin	3×10^{-8}
Cyanocobalamin (vitamin B_{12})	5×10^{-7}
Methylamide of cobalamin‡	8×10^{-6}
Anilide of cobalamin‡	8×10^{-6}
Cobamide coenzymes§	
Adeninyl-	2×10^{-6}
Benzimidazolyl-	5×10^{-5}
5,6-Dimethylbenzimidazolyl-	4×10^{-5}
Non-corrinoids	
$CoCl_2$	1×10^{-3}

* Kindly donated by V. M. Clark, University Chemical Laboratory, Cambridge, England. † Prepared by K. Bernhauer. ‡ Kindly donated by E. L. Smith, Glaxo Laboratories, Ltd., Greenford, England. § Kindly donated by H. A. Barker.

TABLE 5. *Specificity of Corrin Requirement (continued)*

Co^{II} porphyrin complexes	
Co-aetioporphyrin*	No activity or inhibition
Co-coproporphyrin I*	No activity or inhibition
Co^{III} complexes	
$Na[Co(versene)NO_2]$†	Inhibitory ($K_I = 2 \times 10^{-6}$ M)
$[Co\ gly_3]$‡	Inhibitory
$[Co\ dg_2\ (NH_3)_2]Cl \cdot 5H_2O$‡	Inhibitory
$K[Co\ dg_2\ (CN)_2]_3/_2\ H_2O$‡	Inhibitory
$NH_4[Co\ dg_2\ (NO_2)_2]$‡	Inhibitory
$H[Co\ dg_2\ (NO_2)]$‡	Inhibitory
$[Co\ dg_2\ py_2]NO_3$‡	Inhibitory

* Kindly provided by A. W. Johnson, University of Nottingham, England. † Prepared according to the method of Schwarzenbach (16). ‡ Kindly provided by N. Maki, Osaka University, Japan.

tion is a fairly specific one, although there are still many types of compounds that have not been subjected to test in this system. For instance, although all of the highly active compounds contain cobaltic ion, it has not been possible to establish that this oxidation state of the complexed cobalt is essential for coenzyme activity. One interesting and complicating aspect of the cofactor requirement is indicated in Fig. 7, which shows that stimulation by cobinamide only occurs in the presence of coenzyme A, as indicated in the left part of the graph; whereas stimulation of the exchange system by relatively large amounts of cobaltous chloride occurs in the absence of coenzyme A. Both reactions are dependent on mercaptoethanol and enzyme.

Since these observations concerning the corrin requirement were initially made using the relatively exotic purine-fermenting microorganism, we examined the CO_2-pyruvate exchange reaction in a number of other microorganisms, shown in Table 6, which have been extensively used for studies on pyruvic acid metabolism. These include two organisms which have been widely used to study the phosphoroclastic reaction, *Clostridium butyricum* (8) and Werkman's strain of *Escherichia coli* (7), and Crookes' stain of *E. coli*, which has been used for the purification of the pyruvic acid oxidase (9). The relative exchange activities of these microorganisms are indicated as μmoles of CO_2 or formate exchanged with pyruvate in 30 min per mg of protein. The CO_2-pyruvate

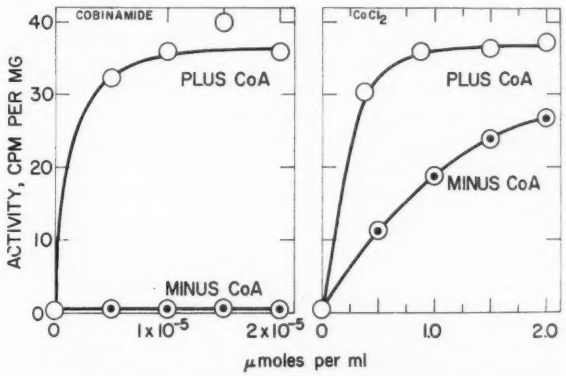


FIG. 7. CoA and corrinoid requirements for exchange reaction. Assay conditions are similar to those described in Table 1, with either cobinamide or $CoCl_2$ added at the concentration shown in the presence or absence of added CoA.

TABLE 6. *CO_2-Pyruvate and HCOOH-Pyruvate Exchange Activities* in Various Bacteria*

Organism	Exchange System		Relative Exchange CO_2/HCOOH
	Pyruvate-CO_2	Pyruvate-HCOOH	
	μmoles/30 min /mg protein		
Clostridium acidi-urici	3.38	0.075	45.1
Clostridium cylindrosporum	1.26	0.030	42.4
Clostridium butyricum	2.80	0.053	53.0
Escherichia coli (Werkman strain)	0.0035	0.034	0.10
Escherichia coli (Crookes strain)	0.012	0.023	0.50

* System contained 55 μmoles of pyruvate, 11 μmoles of $NaHCO_3$ or HCOONa, CoA, cobinamide, and variable amounts of carbonate-C^{14} or formate-C^{14}.

TABLE 7. *Cofactor Requirements for Exchange Activity*

Organism	Exchange Reaction: Pyruvate-	Additions			
		None	CoA	CoA + cobinamide	Boiled extract
		cpm/mg hydrazone			
Clostridium acidi-urici	CO_2	0.97	2.2	80.7	56.0
Clostridium butyricum	CO_2	0.78	0.76	28.1	23.5
Escherichia coli (Werkman)	HCOOH	0.88	1.35	54.3	
Escherichia coli (Crookes)	CO_2	1.2	1.2	10.1	6.9

exchange is about 50 times more active than the formate-pyruvate exchange in the clostridia. Although the amount of formate exchange observed in the *Escherichia coli* strains is comparable to the activity observed in the clostridia, these bacteria are relatively inactive in catalyzing a CO_2-pyruvate exchange. The dependence of the exchange reactions on various cofactors is shown in Table 7. Dowex-treated extracts of the organisms were used. Variable amounts of C^{14} were used to compensate for the variation in exchange activity shown with the different organisms. The activities recorded here are not to be compared from one organism to the other. However, it is clear that the CO_2- or formate-pyruvate exchange activity obtained with each of these organisms shown here is dependent on the addition of a boiled extract, and that the boiled extract requirement can be completely or partially replaced by the addition of coenzyme A and cobinamide but not by CoA alone.

These studies indicate the general nature of the role of the cobinamide and other vitamin B_{12} derivatives in the pyruvate exchange reactions catalyzed by bacterial extracts. The nature of the requirement, and the mechanism by which the corrin derivatives participate in these reactions, remain to be determined.

ADDENDUM

J. Peel (*J. Biol. Chem.*, in press) has recently shown that vitamin B_{12} and its derivatives catalyze the oxidation of mercaptoethanol and other thiols at pH 7.0. The activity of the various vitamin B_{12} derivatives in catalyzing the chemical oxidation roughly parallels the activity of these compounds in the enzymic test reported here. It was further shown that the requirement for vitamin B_{12} derivatives in the CO_2-pyruvate exchange reaction under the conditions described in this communication can be completely overcome when the reaction is performed under anaerobic conditions using extracts of the rumen bacterium LC, strain 1. Similar results have now been obtained by us with extracts of *Clostridium acidi-urici*. It thus seems probable that the activity of the vitamin B_{12} derivatives in the reactions described here is due to their activity in removing oxygen through catalysis of the nonenzymic oxidation of added sulfhydryl derivatives. However, it is also possible that vitamin B_{12} has a more direct effect on the oxidation of enzyme sulfhydryl groups.

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Reaction mechanism of liver alcohol dehydrogenase

HUGO THEORELL

Department of Biochemistry, Nobel Medical Institute, Stockholm, Sweden

FOR SOME 12 YEARS, the liver alcohol dehydrogenase (LADH) has been the subject of my constant, if somewhat intermittent, interest. This is because it offers some attractive properties, making it especially suitable for penetrating studies of its working mechanism. For example, it can rather easily be crystallized in a pure state (2); it is very stable; the combination of LADH with DPNH gives a shift of the maximum absorption from 340 to 325 mμ (6); this shift is accompanied by considerable increase in fluorescence intensity (3); and the formation of ternary complexes with inhibitors is accompanied by further changes in fluorescence intensities and emission maximum (10). Also, as will be described here, its kinetic rate constants agree with equilibrium data, and the two binding sites per molecule operate entirely independent of one another, which simplifies the mathematical treatment of all data to a very considerable extent.

I have recently, and, partially in collaboration with Dr. John McKinley McKee and also in part with Dr. Robert Plane, made a rather extensive study of the kinetics and equilibria in the LADH system, with and without inhibitors of different kinds. Some of the results will be commented upon here.

The general mechanism may be schematically represented as:

(*A*)

where E = ½ LADH, R = DPNH, O = DPN, S = oxid. substrate (aldehyde) and S′ = red. substrate (alcohol).

This formula was found to be correct, since the existence of all the binary and ternary complexes with substrates could be proved using substrate analogues working as inhibitors; and the complexes ER and EO were known before, requiring only more accurate determinations of the dissociation constants, particularly for EO.

However, the total reaction sequence contains not less than 18 different rate constants, and kinetic measurements of initial velocities cannot give more than a maximum of 8. Therefore, simplifications had to be tried, assuming preference for some pathways, and the interconversion between the ternary complexes to be rapid enough not to be rate-limiting.

In 1951, we (7) made a tentative assumption of a reaction sequence

$$E + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ERI \qquad (1)$$

$$ER + S \underset{k_3'}{\overset{k_3}{\rightleftharpoons}} EO + S' \qquad (2)$$

$$EO \underset{k_1'}{\overset{k_2'}{\rightleftharpoons}} E + O \qquad (3)$$

The steady state treatment leads to the equation:

$$\frac{e}{v} = \frac{1}{k_2'} + \frac{1}{k_1[R]} + \frac{1}{k_3[S]} + \frac{k_2}{k_1[R] \times k_3[S]} \qquad (4)$$

The kinetic data of Theorell, Nygaard and Bonnichsen (9) came out in rough agreement with the simplified mechanism; that is, the dissociation constants for ER and EO and the over-all equilibrium constant of the whole reaction sequence were roughly the same, when calculated from the kinetic constants as the value obtained by direct equilibrium discriminations. However, some doubt persisted (10), so still more accurate determinations were desirable. These have now been made at pH 7 and pH 9 (8), with the following results:

The kinetic determinations were made by fluorometric determinations of the initial reaction velocities with systematic variations of the concentrations of DPNH, aldehyde, DPN and alcohol, as described by Dalziel (4). The equilibrium values $K_{E,R}$ and $K_{E,O}$ were determined by spectrophotofluorometrical titrations of LADH with DPNH, without and with DPN present. The K equilibrium was determined by Bäcklin (1). The values of $K_{E,O}$ were now found to be considerably higher than in earlier experiments depending on an earlier unknown, slight quenching effect of DPN on the fluorescence of LADH-DPNH. This effect plays a role at neutral or acid reaction, where high concentrations of DPN have to be used for effective competition with DPNH, but is unimportant at alkaline reaction. This was the cause of the discrepancies earlier considered to obtain at the lower pH values.

It may now be considered as established that the simplified reaction scheme (*eq. 1–3*) and the steady state *equation 4* describe the reaction sequence from a kinetical point of view, even if the true sequence is much more complicated (*scheme A*).

This shows that:

1) ERS → EOS′ is rapid enough not to interfere.

2) EOS′ → EO + S′ is much faster than EOS′ → ES′ + O. In both the forward and backward reaction, the difference in dissociation constants is 30-fold in favor of this sequence.

3) The rate constants for E + R → ER must be ≈ES + R → ESR, and so must E + O → EO and ES′ + O → ESO. It was surprising and by no means self evident why this should be so. However, in studies of ternary complexes enzyme-coenzyme-inhibitors it was found both for fatty acids (caprate), amides (isobutyro-), and for imidazole that the changes in dissociation constants occurring through the addition of a third ligand to a binary complex are paralleled by changes in the dissociation velocity constants, but leaving the association velocity constants essentially unchanged. We have found no exception from this rule in the cases where changes of coulombic attraction forces are not involved. These observations explain why a compulsory order of reaction, as in *equations 1* and *3*, can be assumed and found to be in agreement with experimental data, without this assumption being strictly valid.

TABLE 1

	From Kinetics		From Equilibria	
	pH 7	pH 9	pH 7	pH 9
$K_{E,R}$ μM	0.28	0.86	0.31	0.65
$K_{E,O}$ μM	141	16	160	12
K_{eq}	0.78×10^{-11}	0.77×10^{-11}	0.90×10^{-11}	0.90×10^{-11}

$K_{E,R}$ = dissociation constant of LADH·DPNH. $K_{E,O}$ = dissociation constant of LADH·DPN. $K_{eq} = \frac{[DPNH][ald][H^+]}{[DPN][alc]}$.

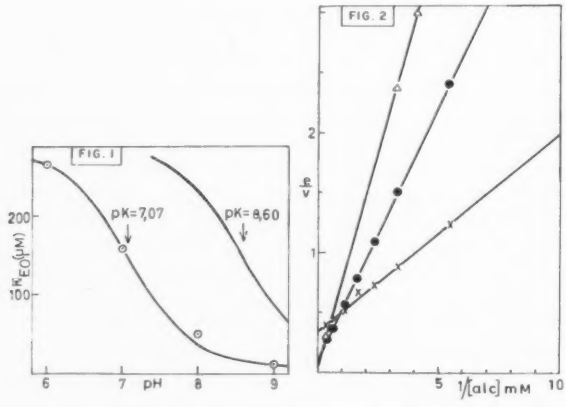


FIG. 1. Experimental values of K_{EO} at pH 6, 7 and 9 adapted to fit a dissociation curve with asymptotes 288 and 8.5 μM, pK 7.07. The titratable group in the free enzyme then has a pK of 8.60.

FIG. 2. Lineweaver-Burk plots with DPN = 255 μM and varied alcohol. pH = 7.0, 23.5°C; X = without imidazole; ● = with 10 mM imidazole; △ = with 20 mM imidazole.

TABLE 2. *Dissociation Constant for Complexes of LADH with Coenzymes and Imidazole (Uncharged, Alcaline Form) μ = 0.1, 23.5° C*

1	2	3	4	5	6	7	8
pH	K_{ER} μM	K_{EI}, mM	$K_{ER,I}$ mM	$K_{EI,R}$ μM	K_{EO} μM	$K_{EO,I}$ mM	$K_{EI,O}$ μM
6	0.23	0.47	4.2	2.0	266	0.8	450
7	0.31	0.55	3.5	2.0	160	1.5	430
8	0.41	0.68	3.6	2.2	51	5.9	440
9	0.65	0.67	3.0	2.9	12	24.2	435

The dissociation constants at pH 6 to 9 are seen in Table 2. Whereas K_{ER} is only moderately sensitive to pH changes, and in this region increases a little with pH, K_{EO} shows a strong effect of opposite sign. As seen from Fig. 1 the values for K_{EO} can be fitted to a monovalent dissociation curve with pK = 7.07 and the asymptotes $K_{EO} \approx 300$ μM at high acidity, $K_{EO} = 8$ μM in the alkaline region. This effect is entirely absent in ER and was, therefore, attributed to a stabilization in alkaline solution of the E − O binding by attraction between the positively charged pyridine ring in DPN^+ and some group in the protein acquiring a negative charge by dissociating a proton in alkaline solution. Our calculations showed that the pK of this group in the protein (without DPN) is around 8.60, which in E-DPN^+ goes down to 7.07 through DPN^+ repelling a proton from this group.

As I shall come back to later, there is good reason to believe that this titratable group at the protein is a water molecule, in acid solution coordinated with zinc. L-ADH contains 2 Zn/mole, and two binding sites for DPNH per mole. We have very strong evidence to show that these operate independently of one another. The hydroxo group, we think, by coulombic attraction stabilizes the bonding of the pyridinium ring of DPN^+ to the enzyme.

EXPERIMENTS WITH INHIBITORS

I shall now tell you a little about our studies on ternary complexes involving enzyme, coenzyme, and inhibitors. Already published work with A. Winer (10) had shown that fatty acids form complexes with E ("EI") and EO ("EOI"), but not with ER. Fatty acids compete in kinetic experiments with alcohol—which was known for formate and acetate since our experiments in 1955 (9)—and also with DPNH, which is a consequence of their inability to form complexes with ER. Fatty acid amides, on the contrary, form ER and ERI complexes, but not EOI, and accordingly compete with aldehydes and DPN.

We recently found, with McKee, that imidazole which is well known to form complexes with Zn ions, makes ternary complexes of both ERI and EOI types. Our spectrophotofluorometric titration method was exceedingly suitable for determining the different dissociation constants, because the ternary complex ERI gives a very

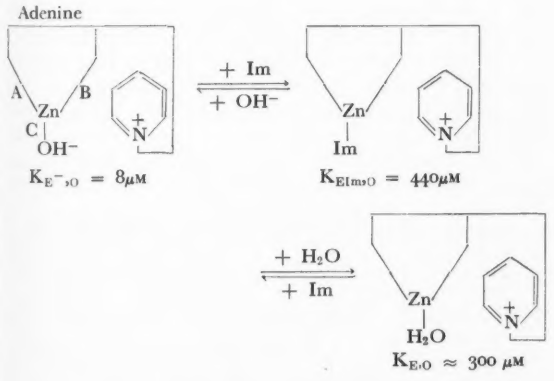
strong fluorescence. At 410 mμ the intensity is 50 times that of free DPNH.

The results are summarized in Table 2.

This table gives (col. 3) the information that one molecule of imidazole is bound to the enzyme in the proportion one to one binding site, which is the same as one per one atom of Zn. The dissociation constant K_{EI} is of the same order of magnitude as for imidazole, forming a coordination compound with free Zn ions. These, however, can attach 4 molecules of imidazole in a tetrahedral complex. Since Zn in the enzyme can attach only one imidazole, this is most easily explained by assuming that three valencies of the Zn are used for binding Zn to the protein. This would give an explanation of the very tight association of Zn with ADH, and why none of the strong bidentate chelators with Zn like ortophenantroline (OP) or α,-α′-dipyridyl, can remove Zn from L-ADH. We came to the hypothesis, that Zn in L-ADH is bound in an octahedral complex with three bonds to the protein and three free for coordinating with coenzymes (adenine part as bidentate ligand, as suggested by Wallenfels and Sund (11), substrates (unidentates) or inhibitors (imidazole, fatty acids, amides: unidentates; OP, α,-α′-dipyridyl, ethylendiamine, glycine: bidentates).

The theory gained considerable support from both kinetic experiments and equilibrium determinations. Some of the kinetic experiments with inhibitors were made in collaboration with Dr. R. Plane, U S A.

1) Imidazole competed with both alcohol and aldehyde, but not with DPNH. With DPN, the kinetics gave nearly competitive pictures. These were surprising, since EOI complexes are formed (see Table 1). This apparent paradox was explained from the data in Table 2, columns 6, 7 and 8, which may be represented in the formulas:



The three free Zn valencies A, B, and C are fixed in a given position on the enzyme surface, and, therefore, not equivalent. Suppose A and B are used for attaching the adenine part of DPN as bidentate, bond C may have either *1)* a hydroxo group, or an alcoholate anion, or a fatty acid anion, or *2)* a neutral imidazole molecule, or *3)* a neutral water molecule. The pyridine ring is known to be close to the adenine part in the natural β-isomer of DPNH, because energy transfer occurs between adenine and pyridine (5, 12). Therefore, the pyridine must, just as the adenine, be close to the zinc atom, and mutual stabilization between the positively charged pyridinium ring in DPN^+, and negatively charged ligands to the zinc will occur. This stabilization effect is 34-fold with the hydroxo group; 23-fold with caprate ion. With alcohol, the situation is complicated by the fact that the ternary complex enzyme—DPN—alcohol undergoes rapid intramolecular conversion. However, a stabilization seems to occur, since the Michaelis constant for DPN goes down 24-fold—when the alcohol concentration is varied from zero to infinity.

2) Fatty acids compete with alcohol for bond C, as already discussed, but they also compete with DPNH. This could have been expected from the fact discovered in collaboration with Winer 2 years ago, that ternary complexes of ADH, DPNH and fatty acids ("ERI") cannot be formed. Fatty acid amides show reverse effects, competing with aldehyde and DPN, and are unable to form EOI complexes. The only logical explanation we have been able to find is that the bonding of the adenine part of the coenzyme makes an "edge shift" on the Zn in connection with oxidation and reduction. Thus, if we denote the valencies occupied by the DPN-adenine A and B, leaving C free for attaching alcohol or fatty acids, the DPNH-adenine should engage, e.g., A and C, leaving B for aldehyde or fatty acid amides.

This theory has been found to be in agreement with all experiments done so far with different inhibitors.

3) Bidentate inhibitors like orthophenantroline, α,-α′-dipyridyl and ethylenediamine, compete with both DPN and DPNH, in agreement with the fact that the adenine part of the coenzymes cannot share three bonds with another bidentate ligand. The two first mentioned inhibitors are strong; ethylenediamine very weak, in agreement with the fact that ethylenediamine as third bidentate ligand in an octahedral Zn complex is known to have a low association constant.

4) Glycine was in equilibrium measurements found to form a compound EI with the enzyme, but neither DPN nor DPNH could then form a ternary complex. This is again, in agreement with the idea that only three valencies of Zn are free, two of them being occupied by glycine as bidentate ligand.

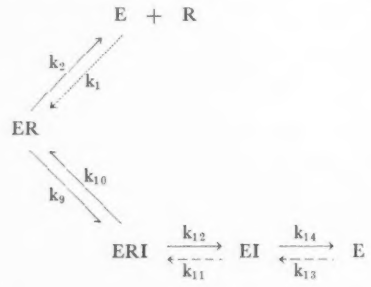
5) At very high alcohol concentrations we found an "unnatural" ER-alc^x complex to be formed; $K^x_{E,alc}$ is = 100 mM, $K^x_{ER,alc}$ = 40 mM and $K^x_{E,alc,R}$ = 0.12 μM. This interesting compound has a lower fluorescence intensity than the natural ER complex. The neutral stabilization (2.5-fold) between R and alcohol explains the 2.5-foid inhibition of the reaction velocity at very high concentrations of alcohol. We think this complex is formed either by alcohol being compelled to use the aldehyde bond, or R the O-bond.

The kinetic experiments with imidazole showed an interesting effect (see Fig. 2). The Lineweaver-Burk plots with and without imidazole sometimes; for example,

when [DPN] was kept high and [alcohol] varied, crossed one another so that at high [alcohol] stimulation, at low [alcohol] inhibition occurred. When the values were extrapolated to infinite imidazole concentration, the reaction velocity at the intercept with the ordinate ([alcohol] = ∞) was stimulated by imidazole by a factor = $K_{EI,R}/K_{ER}$. This is explained in the following way: At high concentrations of both DPN and alcohol, without imidazole, the reaction

$$ER \xrightarrow{k_2} E + R$$

is rate-limiting. When imidazole is present the ternary complex ERI is formed:

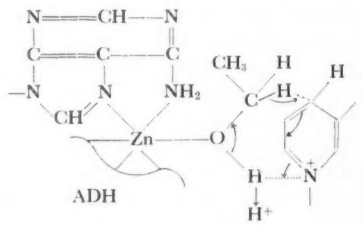


The experiment recorded in Fig. 2 showed a 6.5-fold stimulation of the rate at [Im] $\rightarrow \infty$. This would happen if $k_{14} \gg k_{12}$ which is very probable and $k_{12} = 6.5 \times k_2$; but

$$\frac{K_{EI,R}}{K_{E,R}} = \frac{k_{12} \times k_1}{k_{11} \times k_2} = \frac{2}{0.31} \quad \text{(see Table 2)} = 6.5$$

Therefore, $k_{11} = k_1$, and the "on velocity" of R to EI and to E are the same. This effect is of considerable interest; it offers a possibility of increasing the reaction velocity many-fold. Other nitrogenous, heterocyclic bases seem to react in the same way, and it may be that such reactions are of physiological importance. Substances like imidazole would have a "homeostatic" function towards the substrate: large concentrations lead to rapid oxidation, but as soon as lower concentrations are reached, inhibition occurs.

Time does not allow me to describe any more of our experiments and results. The conclusion on the working mechanism of the liver ADH system could be summarized in the formula:



Summary

As a summary of the most important points of our results I should mention the following:

1) The system reacts according to the general mechanism of an enzyme with two substrates (*scheme A*). The reasons why the simplification made by Theorell and Chance in 1951 is nevertheless valid have been found.

2) Zinc is the activating center, coordinating coenzymes and substrates by the aid of three free valencies of an octahedral complex, the three other bonds being used for the strong attachment of Zn to the protein.

Coenzymes and substrates are restricted to use particular Zn bonds. These restrictions may depend upon additional binding sites, which are certainly operating for both substrates (lipofilic) and DPNH (possibly—SH to the pyridine ring), and DPN^+ (pyridinium attraction to negatively charge group on third Zn-bond). In addition, other steric and space factors very probably play a role. In the interconversion ER ald $\rightleftharpoons$ EO alc, the bonding of both coenzymes and substrates to Zn is shifted ("edge shift"). This explains how nature has accomplished to invent a mechanism making the formation of the useless ternary complexes ER alc and EO ald difficult or impossible.

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Role of thiamin diphosphate in catalysis[1]

L. O. KRAMPITZ, I. SUZUKI,[2] AND G. GREULL

Department of Microbiology, Western Reserve University School of Medicine, Cleveland, Ohio

ALTHOUGH THIAMIN WAS THE FIRST VITAMIN of the B series to be discovered, information concerning its mode of action in enzymatic reactions has only recently become available. It is well known that thiamin diphosphate (TDP), and not thiamin, is the active form of the vitamin in these processes. The term "acetaldehyde-thiamin diphosphate" has been used to describe the unknown nature of the two-carbon moiety, which is presumably involved in pyruvate metabolism, while "active glycolaldehyde" or "glycolaldehyde-thiamin diphosphate" is used to describe the two-carbon moiety involved in transketolase reactions.

Several theories have been advanced to explain the mechanism of action of the coenzyme. Langenbeck (18) proposed a Schiff's base intermediate between pyruvate and the amino group in the 4 position of the pyrimidine moiety of TDP. Lipmann (19) suggested an oxidation-reduction mechanism involving the quaternary nitrogen of the thiazole moiety. Ingraham and Westheimer (11), as well as Breslow (1), proposed that carbanion formation on the N-methylene group of thiamin was involved. The pseudo-base form (20) and the sulfhydryl form (14) of TDP have also been implicated. None of these mechanisms has been experimentally verified with model or enzymatic systems.

Two important observations made with a non-enzymatic system contributed suggestions for a rational approach to elucidation of the mode of action of the coenzyme. Mizuhara and co-workers (21, 22) observed that thiamin and pyruvate, when adjusted to pH 8.8, formed acetoin and that the thiamin was acting catalytically. This non-enzymatic system is remarkably similar to bacterial systems, which form acetoin from pyruvate, and are dependent upon TDP, with the exception that the rate of reaction in the model system is much slower. α-Acetolactic acid has been shown to be an intermediate in the formation of acetoin from pyruvate by both systems, while acetaldehyde cannot be detected (12, 15, 26).

The following general equations have been employed to describe the formation of acetoin from pyruvate:

$$CH_3 \cdot CO \cdot COOH + TDP \rightarrow \text{"}CH_3 \cdot CHO{-}TDP\text{"} + CO_2$$

$$\text{"}CH_3 \cdot CHO{-}TDP\text{"} + CH_3 \cdot CO \cdot COOH \rightarrow$$

$$CH_3 \cdot CO \cdot \underset{\displaystyle OH}{\overset{\displaystyle CH_3}{\underset{|}{\overset{|}{C}}}} - COOH + TDP$$

$$CH_3 \cdot CO \cdot \underset{\displaystyle OH}{\overset{\displaystyle CH_3}{\underset{|}{\overset{|}{C}}}} - COOH \rightarrow CH_3 \cdot CO \cdot CHOH \cdot CH_3 + CO_2$$

$$\text{Sum: } 2CH_3 \cdot CO \cdot COOH \rightarrow CH_3 \cdot CO \cdot CHOH \cdot CH_3 + 2CO_2$$

In contrast to the enzyme systems obtained from bacterial sources, the non-enzymatic system will also form acetoin from acetaldehyde. α-Acetolactate is not an intermediate under these conditions. Presumably the two-carbon moiety involved in the formation of α-acetolactate and acetoin is an adduct with TDP.

The second important obse vation was made by Breslow (2). He found that the hydrogen at position two of the thiazole ring of thiamin exchanged with deuterium of deuterium oxide under conditions of the non-enzymatic system. (Fig. 1. The hydrogen at position two has been encircled). He compared the function of thiamin in the non-enzymatic acetoin forming system to the catalytic function of HCN in the formation of benzoin from benzaldehyde. Breslow (3) and Yount and Metzler (27) also demonstrated that several thiazolium salts other than thiamin catalysed the formation of acetoin from pyruvate under conditions employed by Mizuhara. Breslow (2) synthesized 2-(α-hydroxybenzyl)3,4-dimethyl thiazolium iodide (Fig. 2), which would be the proposed intermediate for benzoin formation from benzaldehyde employing 3,4-dimethyl thiazolium iodide as the catalyst, and found it was not converted to benzoin. Apparently the intermediate was labile and was converted to benzylaldehyde and the original thiazolium salt.

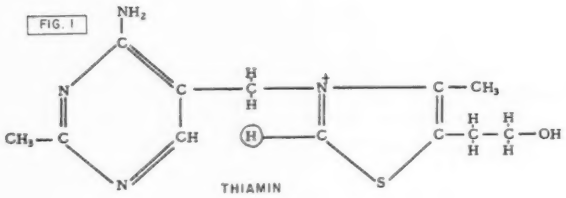
Krampitz et al. (16) synthesized DL-3-[(2-methyl-4-amino - 5 - pyrimidyl) methyl] - 2 - (1 - hydroxyethyl)-4-methyl-5-(2-hydroxyethyl)-thiazolium chloride hydrochloride,[3] the proposed intermediate in acetoin formation from pyruvate (Fig. 3). It will be recognized that the compound, according to the hypothesis presented by Breslow, results from an initial ketol condensation of py-

[1] This investigation was supported in part by a research grant (E-253) from the Public Health Service, and under contract AT(30-1)-1050 with the Division of Biology and Medicine of the Atomic Energy Commission.

[2] Present address: Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan.

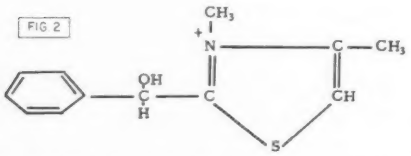
[3] Abbreviated nomenclature: α-hydroxyethyl thiamin (HET)

FIG. 1



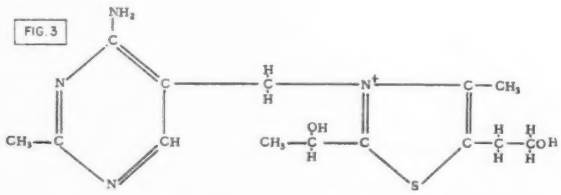
THIAMIN

FIG. 2



2 - (α-hydroxybenzyl) 3, 4-dimethyl thiazolium iodide

FIG. 3



α-hydroxyethyl thiamin (HET)

ruvate with position two of the thiazole ring of thiamin followed by a decarboxylation. We established that the compound replaced thiamin in microbial nutrition experiments and with ATP substituted for TDP in yeast carboxylase preparations containing thiaminokinase. Krampitz *et al.* (17) also showed that HET and acetaldehyde adjusted to pH 8.8 formed acetoin non-enzymatically, while similar quantities of thiamin and acetaldehyde under the same conditions did not form acetoin. Breslow and McNelis (4) synthesized 2- (α-hydroxyethyl)3-benzyl-4-methyl thiazolium bromide and demonstrated it formed acetoin under conditions of the model chemical system.

These results are strongly indicative that HET is the active intermediate in acetoin formation. However, the chemical model systems do not provide proof for the enzymatic processes. The nutritional and enzymatic experiments with HET referred to previously may be criticized on the basis that the catalytic amounts of the compound required in these experiments may have broken down to thiamin by a nonspecific reaction and the activities observed were due to thiamin.

Proof of the intermediary role of an α-hydroxyethyl substitution at position two of the thiazole ring depended upon the synthesis of hydroxyethyl thiamin diphosphate (HETDP) and conversion of substrate quantities of it to specific end products by various enzymes which catalyse pyruvate reactions.

HETDP has been prepared and shown to be an intermediate in the enzymatic reactions illustrated by the following equations:

(1) $CH_3 \cdot CO \cdot COOH \rightarrow CH_3 \cdot CHO + CO_2$

Formation of acetaldehyde from pyruvate by wheat germ pyruvic carboxylase.

(2) $2CH_3 \cdot CO \cdot COOH \rightarrow CH_3 \cdot CO \cdot CHOH \cdot CH_3 + 2CO_2$

Formation of acetoin from pyruvate by the acetoin forming complex obtained from *Aerobacter aerogenes.*

(3) $CH_3 \cdot CO \cdot COOH + 2Fe(CN)_6^{3-} + H_2O \rightarrow CH_3 \cdot COOH + 2Fe(CN)_6^{4-} + 2H^+ + CO_2$

Formation of acetate from pyruvate by a simulated pyruvic oxidase preparation.

In addition α,β-dihydroxyethyl thiamin diphosphate (diHETDP), which by analogy to HETDP would be the glycolaldehyde-TDP intermediate in transketolase reactions, has been prepared and shown to be an intermediate in the formation of sedoheptulose-7-phosphate by transketolase.

PREPARATION OF HETDP

The chemical methods for the phosphorylation of thiamin to TDP are drastic, and we assumed that HET would not be stable under these conditions for phosphorylation to HETDP. Fortunately, however, Dr. C. S. Miller (private communication) has prepared several adducts of thiamin obtained by incubating carbonyl compounds with thiamin under conditions similar to those employed by Mizuhara. With acetaldehyde and thiamin, HET was obtained in yields which approximated 30 to 40% of the original thiamin concentration. HET can also be isolated from mixtures of pyruvate and thiamin at pH 8.8.

Since TDP will substitute for thiamin in the non-enzymatic formation of acetoin from either pyruvate or acetaldehyde, we attempted to isolate HETDP from mixtures of 1-2 C^{14}-acetaldehyde and TDP at pH 8.8. With the aid of paper chromatography, employing *n*-propanol-0.5 M ammonium formate (pH 5.0)-water (65:15:25) as the solvent, a radioactive compound containing TDP, whose specific activity was identical to the acetaldehyde, was isolated. Proof that the material was HETDP consisted of treating the material with prostatic phosphatase and comparing the product with authentic HET. The C^{14}-HETDP was employed in the enzymatic experiments to be described.

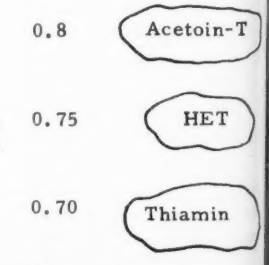


FIG. 4. Chromatographic separation of thiamin and adducts.

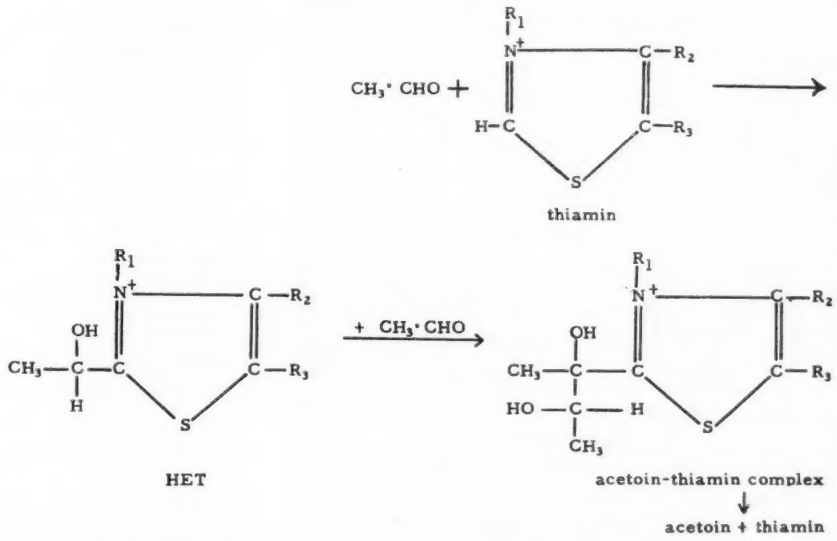


FIG. 5 Mechanism of formation of acetoin. R_1 = pyrimidyl methyl of thiamin; R_2 = $-CH_3$; R_3 = $-CH_2 \cdot CH_2OH$.

A detailed examination of the products formed when thiamin and acetaldehyde were incubated at pH 8.8 gave a greater insight into the mechanism of acetoin formation. Chromatographic separation of such mixtures (Fig. 4) demonstrated not only the formation of HET but also an acetoin complex of thiamin. Figure 5 outlines the mechanism of formation of acetoin. After the initial condensation of acetaldehyde with position two of the thiazole ring to form HET, the latter undergoes an aldol condensation with a second mole of acetaldehyde to form an acetoin-thiamin complex. The latter dissociates to form acetoin and thiamin. With acetaldehyde and TDP at pH 8.8, the acetoin adduct with TDP has also been isolated. This mechanism of an acceptor aldehyde for the so-called acetaldehyde-TDP complex differs from that previously envisioned for enzymatic reactions involving pyruvate, wherein acetoin or α-acetolactate (depending upon the nature of the carbonyl acceptor) were presumably formed without complexing with TDP. Unsuccessful attempts were made to isolate the pyruvic acid adduct with thiamin at position two of the thiazole ring from mixtures of thiamin and pyruvate at pH 8.8. Recognizing that the adduct in all probability would be extremely labile, i.e., decarboxylation to HET, various mild procedures were employed for its identification. In all cases HET and the acetoin adduct with thiamin were the only compounds detected.

Neither the acetoin nor the α-acetolactate complex with TDP has been isolated from enzymatic mixtures employing pyruvate as the substrate. However, we have observed that the acetoin-thiamin and the acetoin-thiamin diphosphate complexes are more labile than HET. The methods employed for the isolation of the acetoin or α-acetolactate-thiamin diphosphate complex from enzymatic mixtures may have destroyed the compounds.

TABLE 1. *Formation of Acetaldehyde from HETDP by Carboxylase*

Exp. No.	Carboxylase, mg	Radioactivity in Recovered Acetaldehyde, cpm
1	0	20
2	1.0	300
3	2.5	500
4	5.0	1100
5	10.0	1800

500 μmoles imidazole buffer pH 6.5; 2 μmoles $MgSO_4$; 5 μmoles C^{14}-HETDP, 15,000 cpm; 10 μmoles sodium pyruvate except in absence of enzyme when pyruvate was replaced with 10 μmoles acetaldehyde. Wheat germ carboxylase resolved of TDP (3,300 u/mg). Total volume 5.0 ml; temp. 30°; time 4 hr. Acetaldehyde collected in 2,4,dinitrophenylhydrazine for counting purposes.

FORMATION OF ACETALDEHYDE FROM HETDP

The first enzymatic study with HETDP was an attempt to liberate acetaldehyde from it with wheat germ pyruvic carboxylase. The pyruvic carboxylase, resolved of TDP, was prepared by a modification of the procedure employed by Singer and Pensky (23). Detection of the acetaldehyde was attempted with the use of alcohol dehydrogenase and DPNH. Oxidation of the DPNH did not occur. Subsequent investigations showed that while the affinity constants of TDP and HETDP for the carboxylase were of the order of 4×10^{-6} M, the kinetics of the dissociation of TDP under the conditions of the experiment were extremely slow. Therefore, the turnover of HETDP and TDP on the catalytic amount of carboxylase employed in the spectrophotometric assay was of such a small order of magnitude we could not possibly detect the formation of acetaldehyde. Relatively large amounts of the enzyme which had been resolved of TDP were, therefore, employed and the release of C^{14} acetaldehyde from substrate amounts of C^{14}-HETDP was determined. These results are depicted in Table 1.

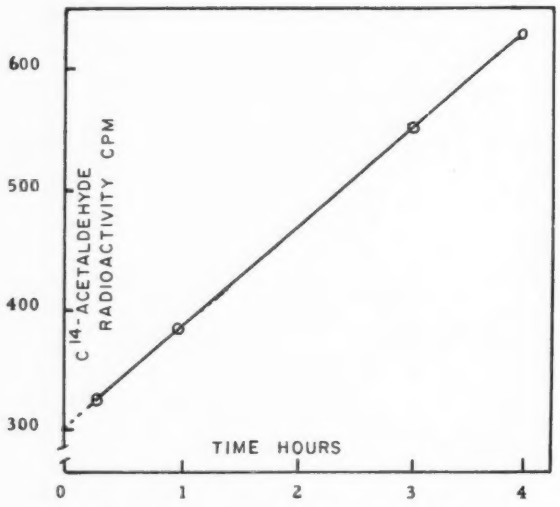


FIG. 6. Formation of acetaldehyde from C^{14} HETDP.

In the absence of enzyme, with 10 μmoles of nonisotopic acetaldehyde added for isolation purposes, the amount of C^{14}-acetaldehyde spontaneously formed from C^{14}-HETDP was negligible. As the amount of enzyme increased from 1 mg to 10 mg, increasing amounts of acetaldehyde were formed and a direct proportionality existed between the amount of carboxylase present and the amount of C^{14}-acetaldehyde formed from C^{14}-HETDP. These results are to be expected because of the slow rate of dissociation of TDP from carboxylase.

Experiments wherein the conditions were similar to those outlined in Table 1 but with a fixed amount of enzyme, 10 mg., showed a linear increase with time of formation of C^{14}-acetaldehyde from C^{14}-HETDP (Fig. 6). Extrapolation to zero time indicated a substantial initial formation of C^{14}-acetaldehyde. These results have been interpreted to mean that the first association of HETDP with the resolved enzyme releases an amount of acetaldehyde which is equivalent to the amount of HETDP required to saturate the enzyme, and the linear rate of release of acetaldehyde which follows is due to the slow dissociation of TDP.

In addition to these experiments which indicate the intermediary role of HETDP in the carboxylase reaction, we have isolated HETDP from a mixture of 20 mg of the purified wheat germ carboxylase and TDP which was forming acetaldehyde from pyruvate. Carlson and Brown (*6*) have recently isolated HET from similar mixtures, after treatment of the phosphorylated compounds with taka diastase.

The action of carboxylase differs significantly from other enzymes which bring about transformations of pyruvate. Acetaldehyde is the major end product of the action of carboxylase on pyruvate, while with the acetoin forming enzyme and pyruvic oxidase acetaldehyde cannot be detected. An explanation of this anomaly is offered by the following equations:

Acetoin formation:

Enzyme (E) + TDP ⟶ E-TDP
E-TDP + pyruvate ⟶ E-TDP-pyruvate
E-TDP-pyruvate ⟶ E-HETDP + CO_2
E-HETDP + pyruvate ⟶ E-TDP-acetolactate
E-TDP-acetolactate ⟶ acetolactate + E-TDP
Acetolactate $\xrightarrow[\text{decarboxylase}]{\text{acetolactate}}$ acetoin + CO_2

When acetaldehyde is present with pyruvate:

E-HETDP + acetaldehyde ⟶ E-TDP-acetoin
E-TDP-acetoin ⟶ E-TDP + acetoin

Carboxylase action:

E-HETDP + acceptor group on enzyme ⟶ $CH_3 \cdot CHO$-E-TDP
$CH_3 \cdot CHO$-E-TDP ⟶ $CH_3 \cdot CHO$ + E-TDP

With all pyruvic enzymes, TDP is bound to the enzyme so that the rate of dissociation of the coenzyme from the protein is extremely slow. The bound HETDP is an initial stable intermediate, formed by the condensation of pyruvate with the E-TDP complex. A decarboxylation occurs forming the E-HETDP complex. For further conversion of HETDP, a second condensation of an acceptor with it must occur. In the case of the bacterial acetoin forming system, pyruvic acid is normally the acceptor forming an α-acetolactate complex with the enzyme bound TDP. The complex dissociates to acetolactate and the E-TDP complex. Acetolactate is decarboxylated to acetoin by a specific decarboxylase which is not TDP dependent. Acetaldehyde, if present with the pyruvate, may combine with the enzyme bound HETDP forming an acetoin complex of TDP which dissociates to acetoin and E-TDP complex.

In the case with carboxylase, evidence has been obtained which indicates that an acceptor group on the protein combines with the enzyme bound HETDP forming an unstable complex which dissociates to acetaldehyde and TDP. In agreement with this concept Juni (13) has recently postulated two sites of action for carboxylase.

FORMATION OF ACETOIN FROM HETDP

To establish the intermediary role of HETDP in the enzymatic formation of acetoin, substrate quantities of the acetoin forming system from *Aerobacter aerogenes* were prepared. The enzyme system was resolved of TDP.

TABLE 2. *Formation of Acetoin from HETDP*

Expt. No.	Enzyme	Radioactivity in Acetoin, cpm	Radioactivity in Residual Acetaldehyde cpm
1	None	75*	0
2	0.3 g (300,000 units)	246	0
3	1.2 g (1,200,000 units)	2170	0

* 5 μmoles nonisotopic acetoin added for isolation purposes. Total volume: 50 ml.; 2 mmoles potassium phosphate buffer, pH 6.5; 40 μmoles $MnCl_2$; 10 μmoles C^{14}-HETDP, 30,000 cpm. 2.5 mmoles acetaldehyde. Time 4 hr; temperature 30°. Acetoin isolated and 2,4-dinitrophenylhydrazone prepared for counting purposes.

$C^{14}H_3 \cdot C^{14}O \cdot CHOH \cdot CH_3$

Table 2 depicts results of an experiment employing large amounts of the purified enzyme, C^{14}-HETDP, and unlabeled acetaldehyde as an acceptor for the hydroxyethyl group. It will be observed that the formation of acetoin from C^{14}-HETDP depends upon the amount of enzyme present. Degradation of the acetoin shows the C^{14} to be exclusively in the acetyl group, which is in agreement with the proposed mechanism.

In addition, HETDP was isolated from a mixture of 22 mg of enzyme, TDP, and pyruvate which was forming acetoin. The formation of acetoin from HETDP and acetaldehyde and the isolation of the intermediate from the enzymatic reaction mixture constitutes reliable evidence for the existence of HETDP as an intermediate in the enzymatic conversion of pyruvate to acetoin.

ENZYMATIC FORMATION OF ACETATE FROM HETDP

The pyruvic oxidase system is a highly organized complex requiring the following cofactors: TDP, lipoic acid, flavinadenine dinucleotide, DPN, and coenzyme A. It has been shown that oxidation of pyruvate will occur in the absence of the oxido-reduction cofactors referred to above if an electron acceptor, such as ferricyanide ion, is employed. Under appropriate experimental conditions, it can be readily demonstrated that the acetoin forming system described previously will form acetate from pyruvate if an electron acceptor, such as the ferricyanide ion or 2,6-dichlorophenol indophenol, is present. Since this acetoin forming complex is devoid of DPN, lipoic acid, coenzyme A, and other components of the normal pyruvic oxidase, one can visualize the mechanism of oxidation of pyruvate in the presence of ferricyanide ion as indicated in Fig. 7.

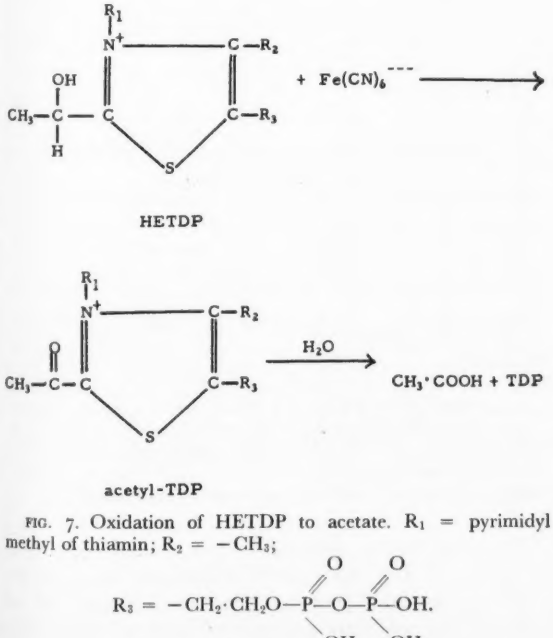


FIG. 7. Oxidation of HETDP to acetate. R_1 = pyrimidyl methyl of thiamin; $R_2 = -CH_3$;

$R_3 = -CH_2 \cdot CH_2O-P(=O)(OH)-O-P(=O)(OH)-OH.$

TABLE 3. *Formation of Acetate from HETDP*

	Radioactivity in Acetate, cpm
Enzyme (845 mg, 5.4×10^6 units)	10,010
No enzyme	657

Reaction mixture contained: 2 mmoles potassium phosphate, pH 6.2; 20 μmoles $MnCl_2$; 10 μmoles C^{14}-HETDP, 130,000 cpm; 200 μmoles potassium ferricyanide; nitrogen in gas phase. Total volume 50 ml; time 2 hr; temp. 30°. 20 μmoles nonisotopic sodium acetate added at conclusion of experiment.

HETDP is formed from pyruvate in a manner analogous to the mechanism in acetoin formation and an enzymatic oxidation of the hydroxyethyl group to an active acetyl group occurs. The latter, in the aqueous environment, is spontaneously hydrolyzed to acetate and TDP. The rate of oxidation of pyruvate is enhanced in the presence of inorganic phosphate and acetyl phosphate can be detected with hydroxylamine. It is likely that phosphorolysis of the active acetyl group at position two of the thiazole ring occurs with the formation of acetyl phosphate. In the pyruvic oxidase complex oxidation of the α-hydroxyethyl group of TDP to an active acetyl group probably occurs by an electron transfer to the oxidized form of lipoic acid with a subsequent acetyl group transfer to dihydro lipoic acid.

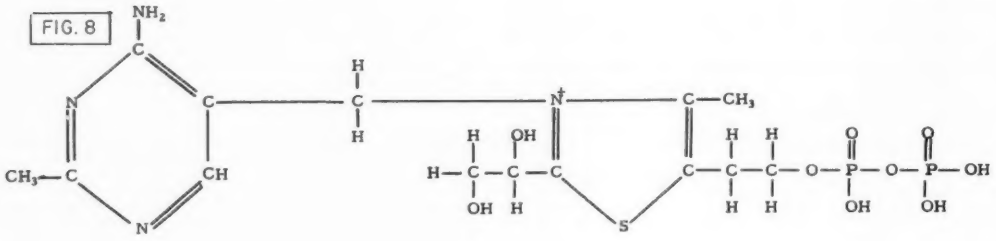
The data depicted in Table 3 demonstrate the oxidation of HETDP to acetate by the acetoin forming system from *Aerobacter aerogenes*, with ferricyanide ion as the electron acceptor. In the absence of enzyme, the oxidation of HETDP by ferricyanide ion as indicated by recovery of C^{14}-acetate was negligible. With 845 mg of enzyme considerable oxidation had occurred. Other experiments showed that over 80% of the oxidation occurred during the first 15 min, indicating that the greater part of the oxidation took place with the initial association of HETDP with the resolved enzyme. Breslow and McNelis (5) and White and Ingraham (25) have very recently demonstrated that an acetyl group substituted at position two of a thiazolium salt was extremely labile in aqueous media. The compound, however, displayed stability in some organic solvents. Holzer and Beaucamp (9) have recently isolated HETDP from a pyruvic oxidase preparation obtained from yeast.

FORMATION OF SEDOHEPTULOSE-7-PHOSPHATE FROM DIHETDP BY TRANSKETOLASE

As mentioned previously, if essentially the same mechanism was involved in transketolase reactions as in pyruvate metabolism, the so-called "active glycolaldehyde" or glycolaldehyde-TDP derivative would be α,β dihydroxyethyl thiamin diphosphate (diHETDP). In the case of sedoheptulose-7-phosphate formation, ribose-5-phosphate would be the aldehydic acceptor for the dihydroxyethyl group of diHETDP. The structure of diHETDP is depicted in Fig. 8.

Attempts to synthesize this compound by reaction of TDP with either glycolaldehyde or hydroxypyruvate at pH 8.8 failed. It will be recalled that during the prepara-

FIG. 8



dihydroxyethyl thiamin diphosphate

(diHETDP)

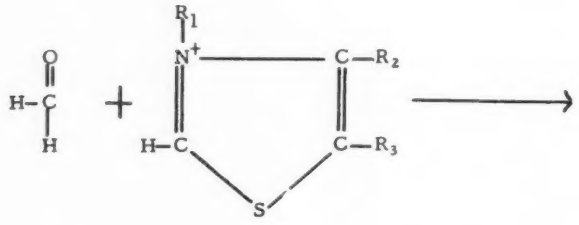
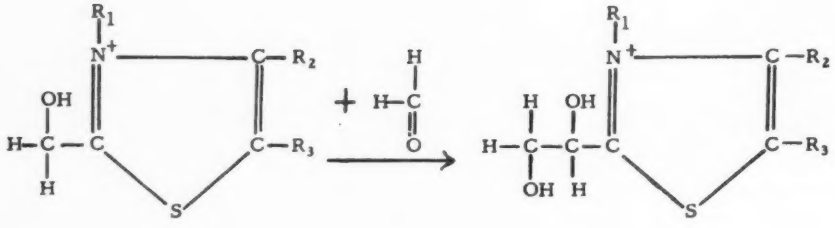


FIG. 9. Preparation of diHETDP. R_1, R_2, R_3 same as Fig. 7.



Hydroxymethyl-TDP **diHETDP**

tion of HET from thiamin and acetaldehyde at pH 8.8, an acetoin complex of thiamin was isolated. This knowledge prompted us to use formaldehyde and TDP at pH 8.8 in an attempt to prepare diHETDP (Fig. 9). The expectation was that hydroxymethyl-TDP would be formed initially from formaldehyde and TDP, followed by condensation of a second mole of formaldehyde to form diHETDP. From such mixtures employing C^{14}-formaldehyde a compound containing TDP was obtained whose specific activity was exactly twice the specific activity of the residual formaldehyde. Furthermore, periodic oxidation of the compound yielded 1 mole of formaldehyde. We assumed that diHETDP had been prepared and proceeded to test it in the transketolase reaction on the basis that if the compound were converted to sedoheptulose-7 PO_4 under the conditions employed, we would have sufficient evidence for proof of structure.

The work by Racker and his co-workers (8) and Horecker and his co-workers (10) have established that

TABLE 4. *Incorporation of C^{14}-di HETDP into Sedoheptulose-7-Phosphate*

	Sedo-7-P isolated, μmoles	Spec. Act.	Total cts.	Spec. Act.	
				C_1	C_2
Transketolase (925 mg S.A. = 5.1)	27.3	612	16,700	154	145
No enzyme	*9.2		110		

50 μmoles C^{14}-diHETDP (3.4×10^5 cpm); 50 μmoles $MgCl_2$; 100 μmoles ribose-5-phosphate; 30 μmoles mixture of xylulose-5-phosphate and ribulose-5-phosphate. pH adjusted to 7.4. Total volume 8.0 ml. Incubated 1 hr at 30°. * 15 μmoles sedoheptulose-7-phosphate added at beginning of experiment.

TDP is not readily dissociable from transketolase. Furthermore, if TDP were freely dissociable from the enzyme, we would expect that with small amounts of the enzyme and substrate amounts of both xylulose-5-PO_4

and TDP, glyceraldehyde-3-PO_4 would be formed in quantities which could be easily detected by a spectrophotometric assay. The so-called glycolaldehyde-TDP would be the other product of the cleavage. Glyceraldehyde-3-PO_4 cannot be detected under these experimental conditions.

Transketolase, which was resolved of TDP, was prepared from baker's yeast by the method described by Srere et al. (24). Table 4 contains data from an experiment wherein large amounts of transketolase were employed with C^{14}-diHETDP, ribose-5-PO_4 as the acceptor, and a pentose phosphate mixture of xylulose-5-PO_4 and ribulose-5-PO_4. In the absence of enzyme, 15 μmoles of sedoheptulose-7-phosphate were added initially and reisolated at the conclusion of the experiment. Practically no C^{14} from the C^{14}-diHETDP appeared in the sedoheptulose-7-phosphate. The sedoheptulose-7-PO_4 which formed enzymatically had a specific activity of 612. Upon degradation, carbon atoms one and two of the sedoheptulose-7-PO_4 contained C^{14}, and each carbon atom had essentially the same specific activity. Although the transketolase preparation used in these experiments was crystalline, it contained traces of ribose-5-PO_4 isomerase and xylulose-5-PO_4 epimerase. With the amounts of enzyme employed under the conditions described, sufficient amounts of these enzymes were present to catalyse an equilibrium mixture of ribose-5-PO_4, xylulose-5-PO_4, and ribulose-5-PO_4. The specific activity of C^{14} in these pentose phosphates was approximately one-half that found in the sedoheptulose-7-PO_4. These results are in accord with the hypothesis that transketolase with TDP catalyses a cleavage of xylulose-5-phosphate to enzyme bound diHETDP and glyceraldehyde-3-phosphate. The former, in all likelihood by a mechanism similar to that outlined for acetolactate formation from pyruvate, forms sedoheptulose-7-phosphate by condensing with ribose-5-phosphate.

Datta and Racker (7) have recently shown that C^{14}-glycolaldehyde is incorporated by an exchange reaction into fructose-6-PO_4 by transketolase catalysis. C^{14}-fructose-6-PO_4 was also shown to incorporate radioactivity into a pool of non-radioactive glycolaldehyde. These investigators have also isolated a glycolaldehyde-enzyme intermediate which transferred the two carbon moiety to ribose-5-PO_4 or free glycolaldehyde with the formation of sedoheptulose-7-PO_4 or erythulose. The enzyme bound glycolaldehyde was postulated to be a glycolaldehyde-TDP derivative. These results are compatible with the concept that TDP is firmly associated with the enzyme and that the di-hydroxyethyl group at position two of the thiazole ring is in an activated state which exchanges with free glycolaldehyde.

SUMMARY

Data have been presented which show that an α-hydroxyethyl substitution at position two of the thiazole ring of TDP is the active intermediate in enzymatic conversions of pyruvate to: *1*) acetaldehyde and carbon dioxide; *2*) acetoin and carbon dioxide; and *3*) acetate and carbon dioxide. In addition it has been shown that an α-β dihydroxyethyl substitution at position two of the thiazole ring of TDP is the active intermediate in reactions catalysed by transketolase.

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Vitamin B_6 coenzymes and mechanism of transamination

W. TERRY JENKINS

Department of Biochemistry, University of California, Berkeley, California

PYRIDOXAL PHOSPHATE WILL COMBINE with many protein molecules, but both the number of molecules bound per mole of protein (14) and the affinities of the binding sites vary (3, 22).

Certain specific proteins, the "pyridoxal enzymes," possess the ability to bind a restricted number of molecules of pyridoxal phosphate in such a manner that the innate ability of the pyridoxal phosphate, itself, to catalyze a particular chemical reaction is increased for certain specific amino acids. This reaction and substrate specificity is not, necessarily, associated with a high affinity of the protein for pyridoxal phosphate. Recently, however, several such enzymes have been isolated with the vitamin still bound to the protein, and this has permitted spectroscopic studies of coenzyme and substrate binding and of the intermediates involved in the catalytic reactions.

SPECTROSCOPIC STUDIES OF THE MODES OF BINDING

Earlier studies of the mode of binding were based largely on the effects of coenzyme analogues upon the activation of the various apoenzymes with pyridoxal phosphate. These studies implicated the phosphate groups, formyl, phenolic and methyl as possible points of attachment to the protein (22).

Pyridoxal phosphate, due to its salicylaldehyde type group, has a yellow color (λ_{max} = 388 mμ) at high pH values but turns colorless at low pH values (18). The characteristic feature of all pyridoxal proteins so far isolated (Table 1) is that they either remain yellow (λ_{max} = 427 mμ) or turn yellow with a maximum at about this wavelength as the pH is lowered (9, 10, 12, 20). This behavior is analogous to that of the pyridoxal valine imines studied by Metzler (15). The imine bond, itself, is very readily hydrolyzed, but is easily reduced with borohydride to the stable secondary amine. Three pyridoxal enzymes have yielded epsilon amino lysine derivatives upon acid hydrolysis after borohydride reduction (5), so that binding of the aldehyde group to this type of amino group may prove to be a general feature.

Actually three different types of color change occur when pyridoxal enzymes are taken to extreme pH values:

1) The spectra may change to those of pyridoxal phosphate itself due to the liberation of the vitamin from the protein. This was first observed by Matsuo and Greenberg with cystathionase (13).

2) The prosthetic group may act as a true pH indicator. This effect was first noted with the glutamic aspartic transaminase and may be a general property of transaminases (9).

3) The bound prosthetic group may isomerize to a different type of bonding. Such an isomerization is suggested by a change in color with pH, which is too rapid to be due to an ionization. Such a rapid change has been noted with phosphorylase and glutamic decarboxylase (20). It is believed that it is due to a conversion of a Schiff base derivative into the uncomplicated Schiff base by a reaction such as that indicated in Fig. 1 (4).

ENZYME-SUBSTRATE COMPOUNDS

It has naturally been suggested that if the aldehyde group is bound to the protein, the amino acid substrate Schiff base intermediates, which are invoked to explain the reactions, could not be formed (1). However, Schiff bases are still chemically analogous to carbonyl compounds, and there is evidence that they may in fact be more reactive. That is to say, the enzyme-substrate compounds are formed bec use the aldehyde group is normally bound in imine linkage to the protein. Water is not liberated in such a reaction (11), and changes in pH would also have little effect, since the group which reacts is similar to that which is liberated. The reaction may in fact resemble that shown in Fig. 1, where X would be the substrate amino group making a nucleophilic attack upon the carbon of the pyridoxal phosphate aldehyde group. With the glutamic aspartic transaminase, it has been observed that the bound pyridoxal phosphate reacts not only with amino acids but also with many carbonyl reagents, virtually instantaneously, even though many of these reagents react only slowly with free pyridoxal phosphate (7).

With an enzyme such as glutamic decarboxylase, the enzyme product complex is so unstable that the reaction rapidly goes to completion and, as with catalase (2), the enzyme-substrate complexes are observed only transi-

torily (21). We were fortunate that with the transaminases the enzyme-substrate complexes could be observed quite readily in the presence of both amino acid and analogous keto acid.

Wada and Snell report that the glutamic aspartic transaminase apoenzyme catalyzes the reversible reaction between free pyridoxamine and the substrate keto acids (23). They found that this reaction was powerfully inhibited by pyridoxal phosphate and pyridoxamine phosphate, which are bound tighter to the same site to reconstitute two forms of the holoenzyme. The two forms of the holoenzyme may also be isolated directly from natural sources, and are shown to be extremely rapidly interconvertible by the addition of either glutamate/ketoglutarate or aspartate/oxaloacetate substrate pairs (8). There is now no doubt that the over-all reaction is as shown in Fig. 2.

Figure 3*A* shows the spectra of the pig heart glutamic alanine transaminase (which we have recently isolated) in the presence of pyruvate alone, alanine alone, and in the presence of an excess of both pyruvate and alanine. In the presence of an excess of both alanine and pyruvate, only the enzyme-substrate complexes can be present. The spectra are complicated, but there appear to be three peaks present at about 330 mμ, 360 mμ, and 490 mμ. The questions that obviously arise are, how many separate species in equilibrium do these three peaks represent, and are they all intermediates in the reaction. It is believed that they do represent three different species for the following reasons, but whether all three are in fact intermediates remains to be demonstrated:

First, when the experiment is performed with glutamate/ketoglutarate, the same peaks appear but the relative intensities differ (Fig. 3*B*). Similar results have been observed with the glutamic aspartic transaminase, but here the effect was even more pronounced. With glutamate/ketoglutarate, one enzyme-substrate complex with a maximum at about 330 mμ predominated. However, with the substrate analogue *erythro*-β-hydroxy-L-aspartic acid, two enzyme-substrate complexes with maxima at 360 and 492 mμ were also observed. It could be shown, moreover, that the complex with λ_{max} 492 mμ preceded the others in the direction from the pyridoxal form of the enzyme (6).

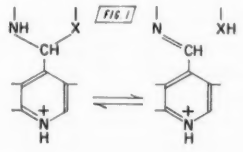
Second, comparison of the spectra of the intermediates

TABLE I

	pH	λ_{max}	pH	λ_{max}	Ref.	Note
		mμ		mμ		
Pyridoxal phosphate	1	295	13	388	18	
Cystathionase	4	430	8.4	424	12	
Glutamic aspartic transaminase	4.6	430	8.4	362	9	pH indicator
Phosphorylase	4.0	415	8.4	333	10	Isomerizes
Glutamic decarboxylase	4.5	420	6.5	335	20	Isomerizes
Glutamic alanine transaminase	4.5	426	8.5	see Fig. 3*A*		pH indicator
Pyridoxal valine imines	8.4	414	12	367	15	

FIG. 1. Reactivity of pyridoxal phosphate Schiff bases.

FIG. 2. Enzyme - substrate reactions of the glutamic-aspartic transaminase.



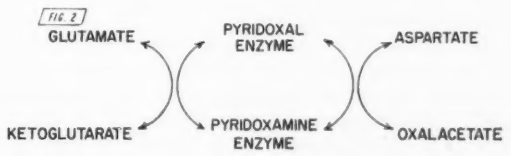
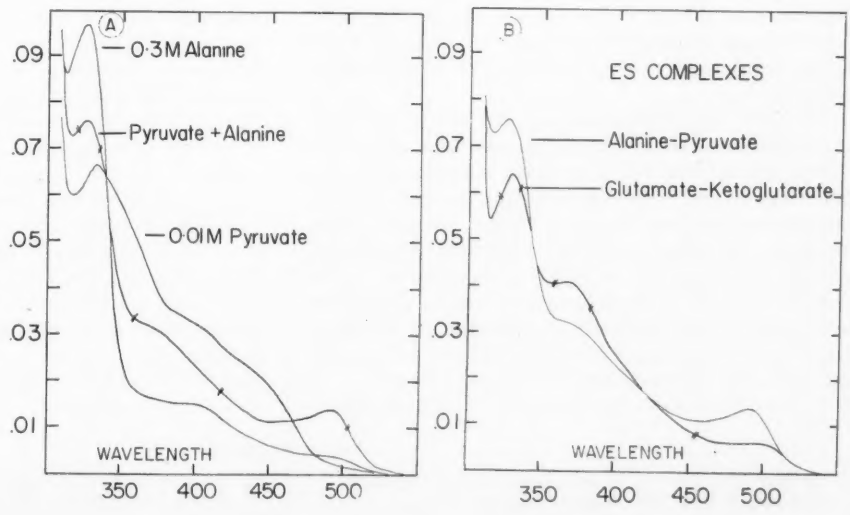


FIG. 3. Enzyme - substrate reactions of the glutamic - alanine transaminase. *A:* Spectra in the presence of alanine and pyruvate alone and with a combination of both. 0.1 M borate pH 9.0. *B:* Enzyme - substrate complexes with 0.3 M L-alanine/0.01 M pyruvate and 0.15 M L-glutamate/0.01 M ketoglutarate. 0.1 M borate pH 9.0.



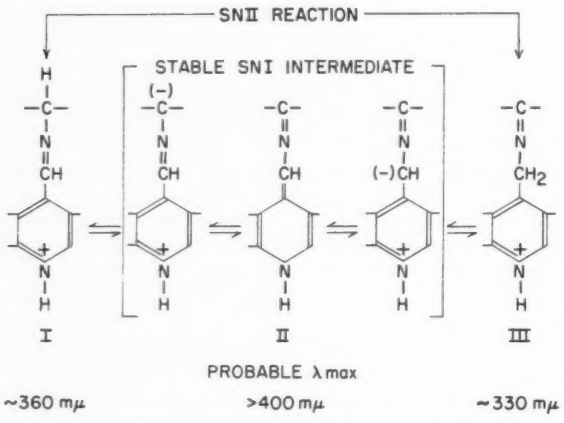


FIG. 4. Structures which have been proposed for the intermediates in transamination.

with those of known pyridoxal phosphate and pyridoxamine phosphate derivatives suggests that the peak at 360 mμ is a Schiff base derivative of the former and the peak at 330 mμ is associated with a derivative of the latter.

NATURE OF THE INTERMEDIATES IN TRANSAMINATION

We would like to know the structure of the compound that absorbs at 492 mμ, since no known pyridoxal phosphate derivatives absorb at such a high wavelength. We should also like to know how the intermediates involved in the reaction are related, and what it is that governs their stability and, hence, makes it possible to observe them spectroscopically.

It is generally believed that the intermediates involved in the reaction are either tautomeric forms, differing, that is to say, in the localization but not the number of their hydrogen atoms; or, alternatively, are conjugate acid/base pairs differing in the number of their protons (19). For example, three of the intermediates commonly believed to be involved in the reaction are shown in Fig. 4, together with their probable absorption maxima. I and III are tautomeric forms, whereas II is their common conjugate base.

Classically two mechanisms are involved in the prototropic rearrangement of azomethine systems such as the conversion of I into III. The direct interconversion is a bimolecular (S_N2) reaction involving catalysis by both proton accepting and donating groups. In many cases, however, a monomolecular (S_N1) rearrangement occurs, a reaction which goes by way of II as intermediate. In the case of pyridoxal derivatives, such an intermediate would be expected to be stabilized by resonance, as indicated, and it has been suggested that this explains the peculiar catalytic activity of pyridoxal phosphate (16, 17).

It might be argued that the existence of three observable enzyme-substrate complexes would support such a series of intermediates, but the following pieces of evidence do not support this conclusion:

1) If the three spectra peaks are to be associated with the intermediates I, II, and III, the peaks associated with complex II should increase in intensity relative to I and III as the pH increases, since II is the conjugate base. Preliminary evidence suggests that the peak at 492 mμ, which might have been associated with II actually decreases by a factor of only 2.5 between pH 6.9 and pH 9.2.

2) On the basis of spectral analogies with the valine imines and the pyridoxal form of the glutamic aspartic transaminase, it seems reasonable to associate the absorbance at 360 mμ with an imine of pyridoxal phosphate, and this has to be the Schiff base involving the amino group of the substrate. The peak at a wavelength as long as 492 mμ must also be due to an imine and, moreover, our evidence suggests that it must precede the one with a maximum at 360 mμ. The derivative with a maximum at 492 mμ may be a tautomeric form of the derivative with $\lambda_{max} = 360$ mμ, which has never been observed in solutions of imines because only the latter tautomeric form predominates in solutions of the vitamin. It is of interest that pyridoxal phosphate imines fluoresce at about 490 mμ, which suggests that this is a characteristic electronic vibration frequency (6, 21).

SUMMARY

At this time several generalizations concerning pyridoxal phosphate containing enzymes appear to be justified.

a) The most characteristic absorption maximum appears to be near 427 mμ. This may be observed, perhaps only transitorily, as the pH is lowered to about pH 4. It indicates that the aldehyde group of pyridoxal phosphate is wholly, or partly, bound to the protein as an imine or imine derivative.

b) Both the type of reaction catalyzed and the substrate specificity reflect differences in the nature of the site binding the pyridoxal phosphate. These differences give rise to unique spectra for the pyridoxal enzymes. Although these spectra vary within wide limits, they are still ascribable to pyridoxal aldimine or imine derivative structures. It is suggested that this particular structural feature is, in fact, a prerequisite for the formation of amino acid Schiff base intermediates.

c) The type of reaction which a pyridoxal enzyme catalyzes is governed by the relative stability of the possible complexes which may be formed. For the same sort of reaction, say transamination, it is likely that the same intermediates occur and that the associated absorption maxima will be similar, even though the equilibrium proportions of the enzyme-substrate complexes vary with the different substrates.

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Biotin enzymes[1]

SEVERO OCHOA AND YOSHITO KAZIRO

Department of Biochemistry, New York University School of Medicine, New York City

NUMEROUS OBSERVATIONS THAT CERTAIN CO_2 fixation reactions or enzymes were decreased in biotin deficiency have long pointed to a function of this vitamin in CO_2 fixation. A function in fatty acid synthesis has also been indicated through the biotin sparing action of oleic acid for certain microorganisms (see Lynen et al. (18) for a review of the literature). A marked advance in our knowledge of the biochemical function of biotin was made when it was found that purified preparations of the enzyme acetyl carboxylase (Table 1, No. 3), which plays an essential role in fatty acid synthesis (6, 24, 25), were rich in biotin and inhibited by avidin (24, 25), the biotin binding protein of egg white. The same was found to be true (1) for β-methylcrotonyl carboxylase (Table 1, No. 2), an essential enzyme in leucine metabolism (1). These observations, and the findings by Lardy and Adler (17) that mitochondrial extracts of biotin deficient rat liver carboxylated propionate at a greatly reduced rate, indicated that biotin must also be the active group of propionyl carboxylase (Table 1, No. 1).

This enzyme is concerned, together with the vitamin B_{12} coenzyme-dependent methylmalonyl isomerase, which converts methylmalonyl CoA to succinyl CoA, with the oxidation of propionic acid (2, 5, 22). Propionyl carboxylase was also found to contain biotin and to be inhibited by avidin (13). Two other biotin enzymes of more recent discovery, transcarboxylase (21, 27) and ATP-dependent pyruvic carboxylase (23), are listed in Table 1. With the exception of transcarboxylase, all of the enzymes in Table 1 catalyze the same kind of reaction, namely a carboxylation driven by ATP which is thereby broken down to ADP and orthophosphate. Since two of these reactions are known to be reversible, it is probable that all reactions of this type are also reversible. Transcarboxylase catalyzes a novel type of reaction involving the intermolecular transfer of a carboxyl group. Methylmalonyl-oxaloacetic transcarboxylase plays an essential role in the conversion of carbohydrate to propionic acid by propionic acid bacteria.

It has been repeatedly observed that biotin is not free in cells and tissues, but bound to protein from which it has to be released by hydrolysis. This has been found to be the case with the biotin enzymes (13, 18, 25). These observations and the fact that a bound form of biotin, biocytin (29), is found in nature, in which biotin is combined by a peptide-like linkage through the carboxyl group of its valeric acid side chain to the ε-amino group of lysine, have led to the belief that in biotin enzymes the vitamin is covalently bound to the protein (18). However, the fact that no biotin is detected before hydrolysis could be explained if the vitamin were dissociably bound to the enzyme with a very low dissociation constant, or if it were part of a large coenzyme molecule, even if this were loosely bound to the protein. Both these possibilities were ruled out by the observation that the biotin content of propionyl carboxylase remained unchanged after precipitation with perchloric acid (13), a procedure which would have split off any compound not covalently bound to the protein. It appears, therefore, likely that biotin is linked to lysine residues in biotin enzymes, but there is so far no direct evidence to support this assumption.

Lynen et al. (18) made the important observation that, on incubation of β-methylcrotonyl carboxylase with large amounts of *d*-biotin in place of β-methylcrotonyl CoA, the free biotin was carboxylated. The compound (biotin $\sim CO_2$) was highly unstable but could be stabilized by conversion to the dimethyl ester with diazomethane. This ester proved to be identical to the dimethyl ester prepared by reaction of *d*-biotin methyl ester with methylchloroformate. The structure of biotin $\sim CO_2$ (18) is shown in Fig. 1, where it is assumed to be bound through a peptide-like linkage to an amino group of the enzyme protein. Of particular interest in relation to the proposed structure is the high instability of biotin $\sim CO_2$ at acid pH values. Thus, Lynen et al. (18) found that 97% of the bound CO_2 was split off within 25 min at pH 4.5 and 0°, while in 0.03 M KOH only 7 and 22% was split in 30 min at 0° and 20°, respectively.

Under conditions similar to those employed by Lynen, and with large amounts of pig heart propionyl carboxyl-

[1] The experimental work reported in this paper was aided by grants from the National Institutes of Health, Public Health Service, and the Rockefeller Foundation. Standard abbreviations, accepted by the Journal of Biological Chemistry, have been used. Although the reacting species of carbon dioxide in the carboxylation reactions catalyzed by biotin enzymes is probably the bicarbonate ion (HCO_3^-), the symbol "CO_2" has been used throughout to refer to this species. The symbol ~ has been used, as is customary, to denote an energy-rich bond as in the expressions CO_2~enzyme, carboxyl~enzyme, or biotin~CO_2.

TABLE 1. *Biotin Enzymes**

No.	Enzyme	Reaction Catalyzed	Mg^{++} requirement	Reference
1	Propionyl carboxylase	ATP + "CO_2" + prop CoA ⇌ ADP + P_i + MMCoA	yes	5, 22
2	β-Methylcrotonyl carboxylase	ATP + "CO_2" + β-CH_3 crot CoA ⇌ ADP + P_i + β-CH_3 glutac CoA	yes	1, 18
3	Acetyl carboxylase	ATP + "CO_2" + acetyl CoA ⇌ ADP + P_i + malonyl CoA	yes	6, 24, 25
4	Transcarboxylase	MMCoA + pyruvate ⇌ prop CoA + oxaloacetate	no	21, 27
5	ATP-dependent pyruvic carboxylase†	ATP + "CO_2" + pyruvate ⇌ ADP + P_i + oxaloacetate	yes	23

* Abbreviations: Prop CoA, propionyl CoA; MMCoA, methylmalonyl CoA; β-CH_3 crot CoA, β-methylcrotonyl CoA; β-CH_3 glutac CoA; β-methylglutaconyl CoA. †A catalytic amount of acetyl CoA or propionyl CoA is required for activity of this enzyme.

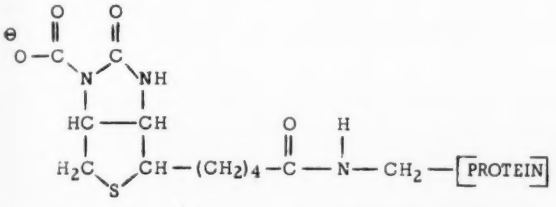
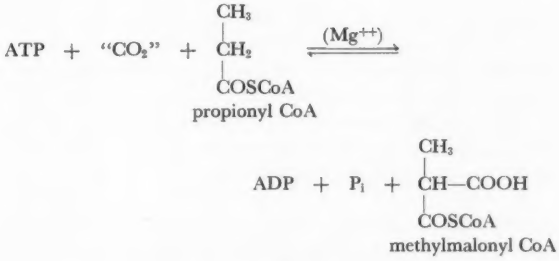


FIG. 1. Structure of enzyme-biotin∼CO_2 (From F. Lynen et al. (18)).

ase, we observed no detectable carboxylation of free *d*-biotin when large amounts of the vitamin were substituted for propionyl CoA. Lynen's results could be explained by the assumption (18) that their enzyme can bind *d*-biotin in a dissociable fashion, and that the "CO_2" combined with biotin covalently bound to the enzyme can be transferred to the dissociably bound vitamin. If so, our negative results would simply reflect a lack of affinity of propionyl carboxylase for free biotin. Another possibility would be that the relatively crude enzyme preparations used by Lynen et al. in these experiments contained enzymes capable of hydrolyzing biotin (or biotin ∼ CO_2) off the carboxylase and of linking biotin to the enzyme in the presence of ATP. There is little doubt as to the biological nature and significance of the carboxylation of biotin with β-methylcrotonyl carboxylase since, as pointed out by Lynen, only the natural *d*-form of the vitamin reacted, the *l*-enantiomorph being inactive. The ensuing discussion will be concerned with recent studies of our laboratory on propionyl carboxylase and the mechanism of the reaction catalyzed by this enzyme.

Propionyl carboxylase catalyzes the reaction:



The stoichiometry and reversibility of this reaction was established in 1959 (22), with highly purified preparations of the enzyme from pig heart. This enzyme has recently been crystallized (14). Photographs of the crystals are shown in Fig. 2. Figure 3 shows sedimentation and electrophoresis patterns of the crystalline enzyme. The enzyme has a molecular weight of 700,000 and contains 1 mole of bound biotin per 175,000 g of enzyme, or 4 moles per mole of enzyme. Some of its physical and catalytic constants are listed in Table 2. It is not unlikely that propionyl carboxylase might consist of four subunits of molecular weight 175,000, each with 1 molecule of biotin. This would be in line with observations made on other enzymes. Thus, crystalline muscle phosphorylase *a*, with a molecular weight of 500,000, is known to be a tetramer of four identical subunits of molecular weight 125,000, each containing 1 molecule of bound phosphate and 1 of pyridoxal phosphate (3, 19). Muscle phosphorylase *a* is reversibly split into four subunits by *p*-chloromercuribenzoate. On ultracentrifugation in 7.0 M urea, propionyl carboxylase yields a single sharp peak with a sedimentation coefficient of 2.5 S instead of 19.7 S for the native enzyme (Fig. 3). This suggests dissociation of the molecule into a number of equal subunits the

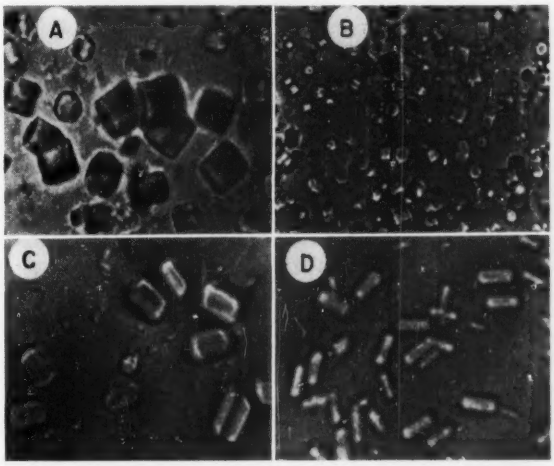


FIG. 2. Crystalline propionyl carboxylase from pig heart (14). *A* and *B* are different magnifications of crystals obtained at room temperature. *C* and *D* are photographs of crystals, at about the same magnification, obtained at 0°. As seen in *C*, these are occasionally rather large.

molecular weight of which has as yet not been determined. However, this treatment led to irreversible inactivation of the enzyme. It is becoming increasingly apparent that other functional proteins, e.g., hemoglobin (11), glutamic dehydrogenase (8), and yeast alcohol dehydrogenase (15), are made up of subunits and are inactive or largely so in the dissociated state.

As is the case for other biotin enzymes, propionyl carboxylase is inhibited by avidin. Of special interest is the fact that, as shown in Fig. 4, close to 100% inhibition is obtained with stoichiometric amounts of avidin after incubation with the enzyme for 10–15 min. This indicates that avidin has as much affinity for enzyme-biotin as it has for the free vitamin, a result which is in line with the observation of Wright et al. (28) that avidin has the same degree of affinity for biotin and biocytin. The purest preparations of avidin have a binding capacity of 8 μg biotin/mg (8 units per mg) and a molecular weight between 60,000 and 70,000 (7). Since their biotin-binding capacity corresponds to 1 mole biotin per 30,000 g avidin, Warner (26) considers it likely that 2 molecules of biotin are bound by 1 of avidin. If so, for a molecular weight of 700,000, 1 molecule of pig heart propionyl carboxylase containing 4 molecules of biotin would bind 2 molecules of avidin. Pig heart propionyl carboxylase is very sensitive to sulfhydryl binding reagents. One hundred per cent inhibition was obtained with 5×10^{-6} M *p*-chloromercuribenzoate (13).

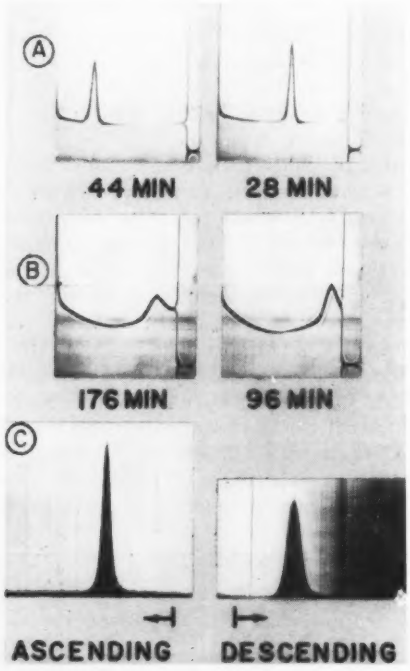


FIG. 3. Sedimentation and electrophoresis patterns of crystalline propionyl carboxylase (14). *A:* Sedimentation in phosphate buffer. *B:* Sedimentation in phosphate buffer containing 7.0 M urea. Electrophoresis in tris buffer at pH 7.5.

TABLE 2. *Physical and Catalytic Constants of Crystalline Propionyl Carboxylase (14)*

Physical Constants	Catalytic Constants	
	Apparent K_m	V_{max} (30°, pH 8.5)
	moles/liter × 10^8	*moles/min/mole enzyme*
Molecular wt., 700,000	ATP,* 0.08	Propionyl CoA, 21,600
Isoelectric point, 6.1	HCO_3^-,* 2.5	Butyryl CoA, 2,500
Bound biotin, 4 moles/mole	Propionyl CoA, 0.2 Butyryl CoA, 1.5	

* Determined with amorphous enzyme (22)

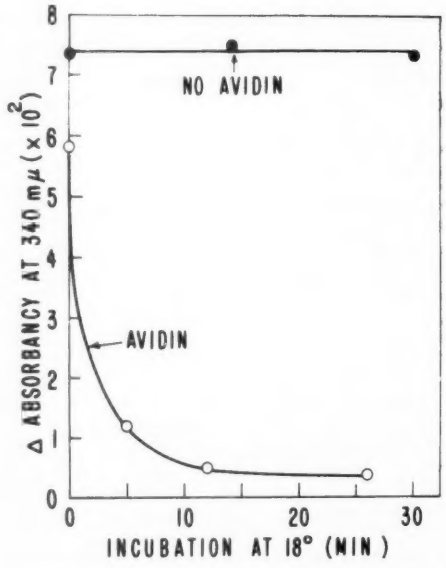


FIG. 4. Time course of inhibition of propionyl carboxylase by a stoichiometric amount of avidin (13). 53 μg of enzyme (with 0.075 μg of bound biotin) were incubated without, or with, 0.075 unit of avidin (1 unit binds 1.0 μg of free biotin (4)) and the enzyme activity assayed at various time intervals.

Several lines of evidence indicate that the propionyl carboxylase reaction involves two separate steps as follows:

(1) ATP + "CO_2" + enzyme $\xrightleftharpoons{(Mg^{++})}$ ADP + P_i + $CO_2 \sim$ enzyme

(2) $CO_2 \sim$ enzyme + propionyl CoA $\rightleftharpoons$ enzyme + methylmalonyl CoA

(3) Sum: ATP + "CO_2" + propionyl CoA $\xrightleftharpoons{(Mg^{++})}$ ADP + P_i + methylmalonyl CoA

The first step (*reaction 1*) would be shared by enzymes 1, 2, 3, and 5 of Table 1.

On the basis of experiments on the exchange of radioactive P_i or ADP with ATP, *reaction 1* was believed (10,

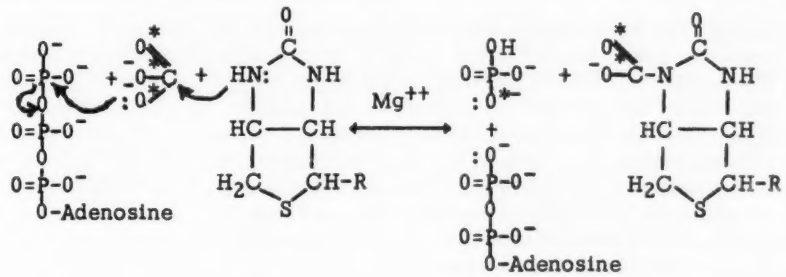


FIG. 5. Possible mechanism of reaction 2. O^{18} label indicated by asterisks.

$$ATP^{4-} + CO_3^{18}\ominus\!\!\ominus + \text{biotin-enzyme} \xleftrightarrow{(Mg^{++})} ADP^{3-} + HPO^{18}O_3^{2-} + CO_2^{18}\ominus \text{ biotin-enzyme}$$

18) to proceed in two substeps as shown by *reactions 1a* and *1b* below.

$$(1a)\ \text{ATP} + \text{enzyme} \xrightleftharpoons{(Mg^{++})} P_i + \text{enzyme} \sim \text{ADP} \quad (\text{or ADP} + \text{enzyme} \sim P_i)$$

$$(1b)\ \text{Enzyme} \sim \text{ADP (or enzyme} \sim P_i) + \text{"}CO_2\text{"} \xrightleftharpoons{(Mg^{++})} \text{ADP (or } P_i) + \text{enzyme} \sim CO_2$$

This is unlikely as careful reinvestigation (13) showed that P_i was required for ATP-ADP exchange and, conversely, ADP was required for ATP-P_i exchange. A requirement for bicarbonate was also evident in these experiments. It then appears that *reaction 1* proceeds in a single step. Boyer has found (unpublished), in experiments with crystalline propionyl carboxylase and H_2O^{18} or $NaHCO_3{}^{18}$, that one O^{18} from the bicarbonate appears in the orthophosphate and two O^{18} atoms appear in the carboxyl group of methylmalonyl CoA. When corrected for the equilibration between CO_2, H_2O, and bicarbonate, there was no incorporation of O^{18} from water. *Reaction 1* may be visualized (Fig. 5) as a concerted reaction involving nucleophilic attack of the biotin nitrogen on the bicarbonate carbon, followed by attack of the bicarbonate oxygen on the terminal phosphate of ATP.

The formulation of the over-all reaction as involving formation of an energy-rich carboxyl ~ enzyme as intermediate, proposed for β-methylcrotonyl carboxylase (18) and propionyl carboxylase (13, 16) on indirect evidence finds direct support in our experiments on the carboxylation and decarboxylation of propionyl carboxylase (12). Following incubation of the enzyme with either ATP and "$C^{14}O_2$", in the presence of Mg^{++}, or with methylmalonyl CoA labeled with C^{14} in the carboxyl group,[2] $C^{14}O_2$ ~ enzyme can be isolated in about 50% yield by passage through a Dowex-1 column. One mole of "$C^{14}O_2$" is taken up per mole of protein-bound biotin or 4 moles per mole of enzyme.

In preliminary experiments on the transfer of "$C^{14}O_2$" from $C^{14}O_2$ ~ enzyme to propionyl CoA, it was found that, on incubation for 5 or more minutes at room temperature, little or no methylmalonyl CoA-C^{14} was present. However, most of the radioactivity of the carboxyl ~ enzyme was found in methylmalonyl CoA when the reaction mixtures were supplemented with ATP and Mg^{++}. In the latter case, a large amount of nonlabeled bicarbonate was also added, since propionyl CoA might be carboxylated not through direct carboxyl transfer from the $C^{14}O_2$ ~ enzyme but, by way of the over-all reaction (*reaction 3*) by "$C^{14}O_2$" arising by decarboxylation of the $C^{14}O_2$ ~ enzyme. These results led us to discuss the possibility that ATP might be required for the transfer of "CO_2" from the carboxyl ~ enzyme to propionyl CoA (13).

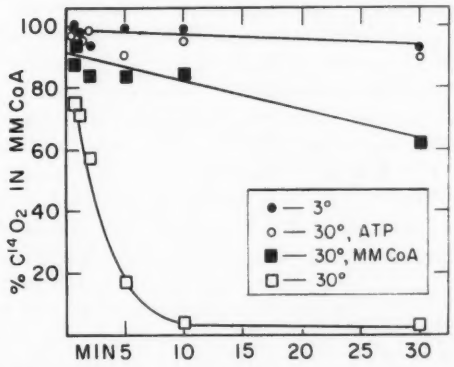


FIG. 6. Influence of various factors on the amount of methylmalonyl CoA-C^{14} found after incubation of $C^{14}O_2$~enzyme with propionyl CoA (12). Carboxylation of the enzyme with ATP + "$C^{14}O_2$" in the presence of Mg^{++}.

Closer investigation showed that labeled methylmalonyl CoA was always found in good yield, in the absence of ATP, provided the incubation was conducted either at low temperature or for a very short time at 25–30°. As seen in Fig. 6, on very brief incubation (½–1 min) at 30° (–□–□–□–), most of the radioactivity of the $C^{14}O_2$ ~ enzyme is recovered in methylmalonyl CoA. However, the recovery rapidly decreases, as the incubation is lengthened, to become negligible after

[2] Methylmalonyl CoA-3-C^{14} was prepared by reaction of ATP, "$C^{14}O_2$", and propionyl CoA, in the presence of propionyl carboxylase, and purified by ion-exchange chromatography (12).

10 min. This result suggests that "$C^{14}O_2$" first transferred from the enzyme to propionyl CoA, is subsequently released due to the reversibility of *reaction 2* and to instability of the carboxyl ~ enzyme at this temperature. On the other hand, at 3° (-●-●-●-) most of the radioactivity originally on the enzyme is found in methylmalonyl CoA even after incubation for 30 min. This indicates that the carboxyl ~ enzyme is rather stable at temperatures near 0° so that an equilibrium is established. It may further be seen (Fig. 6, -○-○-○-), that ATP prevents the loss of methylmalonyl CoA radioactivity at 30°. The "protective" effect of ATP is apparently due to the fact that, in the presence of an excess propionyl CoA and nonlabeled bicarbonate, a large amount of cold methylmalonyl CoA is produced with considerable dilution of the methylmalonyl CoA-C^{14} formed by the rapid transcarboxylation between $C^{14}O_2$ ~ enzyme and propionyl CoA. Under these conditions, instability of the carboxyl ~ enzyme leads to loss of "$C^{14}O_2$" of very low specific radioactivity and, therefore, to a very slow loss of methylmalonyl CoA counts. This explanation is borne out by one other experiment in Fig. 6 (-■-■-■-). When a moderate excess of cold methylmalonyl CoA is present in a sample containing $C^{14}O_2$ ~ enzyme and propionyl CoA, methylmalonyl CoA radioactivity, which as usual is high after half a minute, decreases rather slowly over a 30-min incubation at 30°. It is, therefore, apparent that the carboxyl ~ enzyme, fairly stable at temperatures in the neighborhood of 0°, is quite unstable at higher temperatures.

If we assume that the decrease in methylmalonyl CoA radioactivity with time, in the experiments of Fig. 6, is due to spontaneous decarboxylation of the CO_2 ~ enzyme, the half-life of the CO_2 ~ enzyme at 30° and pH 7.5 (Fig. 6. -□-□-□-) would be about 3 min.

TABLE 3. *Carboxylation and Decarboxylation of Propionyl Carboxylase. Carboxylation by ATP + "$C^{14}O_2$" (12)*

Carboxylation		Decarboxylation			
ATP + "$C^{14}O_2$" + E $\xrightarrow{(Mg^{++})}$ ADP + P_i + $C^{14}O_2$ ~ E		$C^{14}O_2$ ~ E + prop CoA → E + MMCoA-C^{14}		CO_2 ~ E + ADP + P_i^{32} $\xrightarrow{(Mg^{++})}$ E + "CO_2" + ATP^{32}	
System 1	Radioactivity of enzyme	System 2	Radioactivity of MMCoA	System 3	ATP^{32} formed
	cpm		*cpm*		*mμmoles*
Complete	11,050	Complete	10,900	Complete	10.7
No ATP	160	No prop CoA	0	No ADP	0.05
		EDTA (0.02 M)	10,280		
		Avidin (11 units)	554		
		PCMB (5×10^{-4} M)	0		

System 1. 1.14 mg enzyme. Incubation, 3 min at 25°. 0.565 mg (ca. 3.2 mμmoles biotin) of $C^{14}O_2$ ~ enzyme were recovered. System 2. Aliquots of $C^{14}O_2$ ~ enzyme from system 1 were used and the results referred to the 0.565 mg recovered. Incubation, 2 min at 3°. System 3. Carboxylation as in system 1 except for the use of cold "CO_2." 1.95 mg (ca. 11.1 mμmoles biotin) CO_2 ~ enzyme. Incubation, 5 min at 3°. Abbreviations as in Table 1. E denotes enzyme (propionyl carboxylase).

TABLE 4. *Carboxylation and Decarboxylation of Propionyl Carboxylase Carboxylation by Methylmalonyl CoA-3-C^{14} (12)*

Carboxylation		Decarboxylation			
MMCoA-C^{14} + E → prop CoA + $C^{14}O_2$ ~ E		$C^{14}O_2$ ~ E + prop CoA → E + MMCoA-C^{14}		$C^{14}O_2$ ~ E + ADP + P_i^{32} $\xrightarrow{(Mg^{++})}$ E + "$C^{14}O_2$" + ATP^{32}	
System 1	Radioactivity of enzyme	System 2	Radioactivity of MMCoA	System 3	ATP^{32} formed
	cpm		*cpm*		*mμmoles*
Complete	20,200*	Complete	19,700	Complete	3.54
		No prop CoA	105	No ADP	0.05
		EDTA (0.02 M)	19,800		

System 1. 1.11 mg enzyme; methylmalonyl CoA-3-C^{14} (7.85×10^6 cpm/μmole). Incubation, 5 min at 0°. 0.51 mg (ca. 2.92 mμmoles biotin) $C^{14}O_2$ ~ enzyme were recovered. Systems 2 and 3 as in Table 2, except that $C^{14}O_2$ ~ enzyme (0.66 mg, ca. 3.77 mμmoles biotin) was used for the latter. * 2.58 mμmoles of "$C^{14}O_2$." Thus, carboxylation of the enzyme (with 2.92 mμmoles of biotin) was almost quantitative. Abbreviations as in Table 3.

The rate of spontaneous breakdown of the CO_2 ~ enzyme has also been measured spectrophotometrically, under the conditions of the optical assay of propionyl carboxylase (22), by using relatively large amounts of enzyme in the absence of propionyl CoA. There is a rather abrupt initial decrease in absorbancy at 340 mμ, actually corresponding to the almost instantaneous carboxylation of the enzyme with release of an equivalent amount of ADP. This is followed by a prolonged, much slower decrease at a linear rate which, in the absence of ATPase activity, presumably reflects the rate of decarboxylation of the CO_2 ~ enzyme. The half-lives of the CO_2 ~ enzyme calculated in this manner were 2.9, 2.6, and 1.6 min at pH 6.4, 8.1, and 9.2, respectively. These values are of the same order of magnitude as the one derived from Fig. 6. Closer comparison of the stability of the CO_2 ~ enzyme with Lynen's biotin ~ CO_2 is not warranted at this time, but the stability of the two compounds may be said to be roughly of the same order of magnitude.

In the experiments of Tables 3 and 4, reactions of the $C^{14}O_2$ ~ enzyme were studied at 3° under conditions in which the carboxyl ~ enzyme is relatively stable. They show that its radioactivity can be quantitatively transferred to propionyl CoA. Like the over-all reaction, this reaction is inhibited by avidin and by *p*-chloromercuribenzoate. Lack of inhibition by ethylene diaminetetraacetate indicates that Mg^{++} is not required for this step. It may be noted in the experiment of system 1 (Table 2), for which the enzyme was carboxylated with methylmalonyl CoA-3-C^{14}, that the enzyme (containing 2.92 mμmoles of biotin) took up 2.58 mμmoles of "$C^{14}O_2$" or almost 1 mole per mole. As further shown in Tables 3 and 4 (system 3), the carboxyl ~ enzyme reacts with ADP and P^{32}-labeled orthophosphate, in the presence

of Mg^{++}, to yield ATP^{32} quantitatively. The above results, then, provide conclusive evidence that the over-all reaction catalyzed by propionyl carboxylase is the result of two partial, reversible reactions, each involving carboxylation and decarboxylation of the enzyme. The enzyme is carboxylated either by "CO_2" + ATP (*reaction 1*, forward) in the presence of Mg^{++}, or by methylmalonyl CoA (*reaction 2*, backward) in the absence of Mg^{++}. The energy-rich carboxyl enzyme either transfers "CO_2" to propionyl CoA (*reaction 2*, forward) in the absence of Mg^{++}, or is decarboxylated by ADP + orthophosphate to form ATP (*reaction 1*, backward) in the presence of Mg^{++}.

Under our experimental conditions, the uptake of "CO_2" by the enzyme approached 1 mole per mole of protein-bound biotin, i.e., 4 moles per mole of enzyme, and the bound "CO_2" was almost quantitatively transferred to propionyl CoA or utilized for the synthesis of ATP from ADP and orthophosphate in a 1 mole "CO_2" per mole of ATP ratio. These conditions obviously favored extensive displacement of the equilibrium position of each partial reaction in the direction investigated. The stoichiometry of carboxylation of the enzyme suggests that the $CO_2 \sim$ enzyme is enzyme-biotin $\sim CO_2$, as proposed by Lynen. This view finds some additional support in the similar degree of instability of the $CO_2 \sim$ enzyme and Lynen's biotin $\sim CO_2$, but further work is required to throw more definite light on this point.

Reaction 2 can be visualized as a transcarboxylation and is undoubtedly a step in the over-all reaction (*reaction 6*), catalyzed by transcarboxylase (27), for the transcarboxylase reaction can be considered, in the light of the above results, the net result of the partial *reactions 4* and *5*.

(4) Oxaloacetate + enzyme $\rightleftharpoons$ pyruvate + $CO_2 \sim$ enzyme
(5) $CO_2 \sim$ enzyme + propionyl CoA $\rightleftharpoons$ enzyme + methylmalonyl CoA
(6) Sum: Oxaloacetate + propionyl CoA $\rightleftharpoons$ pyruvate + methylmalonyl CoA

Halenz and Lane (9) have recently presented evidence that propionyl carboxylase can function as a transcarboxylase yielding methylmalonyl CoA-C^{14} on incubation with ethylmalonyl CoA and propionyl CoA-C^{14}. This is due to the fact that, as shown by several authors (14, 16, 20), propionyl carboxylase reacts slowly with butyryl CoA to form ethylmalonyl CoA. On the basis of the results presented here, this transcarboxylation can be explained by the coupling of *reaction 7* with *reaction 5*

(7) Ethylmalonyl CoA + enzyme $\rightleftharpoons$ butyryl CoA + $CO_2 \sim$ enzyme

All biotin enzymes which, like enzymes 1, 2, 3, and 5 in Table 1, catalyze ATP-dependent carboxylations are probably "double-headed" two-step reaction enzymes. The first step leads to carboxylation of the enzyme (presumably to form enzyme-biotin$\sim CO_2$) with liberation of ADP and orthophosphate. This step would be common to all of them. The second, substrate-specific step is a transcarboxylation from the $CO_2 \sim$ enzyme to the "CO_2" acceptor. Both steps are probably reversible in all cases. Transcarboxylase is so far the only example of a biotin enzyme which brings about not an uptake of "CO_2", but exclusively a carboxyl transfer from one molecule to another via a two-step, reversible reaction involving intermediate carboxylation of the enzyme. All of the known biotin enzymes are transcarboxylases, but those of them which bring about an uptake of free "CO_2" have an additional, ATP-utilizing mechanism built into them to make this possible.

The pyruvic carboxylase of Utter and Keech (23) is a curious enzyme which may combine the features of transcarboxylase and the ATP-dependent carboxylases. Carboxylation of pyruvate by this enzyme requires, besides ATP and Mg^{++}, catalytic amounts of either acetyl CoA or propionyl CoA. It is of interest in this connection that, while "CO_2" is not directly transferred from propionyl carboxylase to transcarboxylase, the two enzymes can be coupled, in the presence of catalytic amounts of propionyl CoA, to convert pyruvate + "CO_2" to oxaloacetate (27).

This reaction (*reaction 10*) is the net result of *reactions 8* and *9*.

(8) ATP + "CO_2" + propionyl CoA $\xrightleftharpoons{(Mg^{++})}$ ADP + P_i + methylmalonyl CoA
(9) Methylmalonyl CoA + pyruvate $\rightleftharpoons$ propionyl CoA + oxaloacetate

(10) Sum: ATP + "CO_2" + pyruvate $\xrightleftharpoons{(Mg^{++})}$ ADP + P_i + oxaloacetate

Thus, the combined action of these enzymes gives the same result that is obtained with the ATP-dependent pyruvic carboxylase.

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BIOLOGICAL ROLE AND MECHANISM OF ACTION OF VITAMIN K

Nutritional hypoprothrombinemia and metabolism of vitamin K[1]

E. A. DOISY, JR.

Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri

UNTIL COMPARATIVELY RECENTLY, deficiency of the K vitamins in normal adult animals had not been recognized. In order to develop hypoprothrombinemia in adult rats, administration of drugs, or antagonists, or surgical alteration of biliary and digestive tracts was necessary. Thus, the disorder was restricted to the newborn or the newly hatched and rapidly growing organism. It is my purpose today to re-examine some of these concepts and time honored precepts in the light of more recent information. Many new facts have been brought to light due in large part to the Army's interest in the adequacy of foods preserved by irradiation. The Symposium[2] published in Federation Proceedings of December 1960 summarizes many of these findings (36). Time does not permit a discussion of the interesting findings on germ-free animals.

The first observation which stimulated so much activity was that of Johnson and his co-workers at the University of Illinois (21, 30). Most of the young male rats fed a diet unsupplemented with vitamin K containing 35% solids of irradiated beef died of hemorrhage. These were observed to occur throughout the animal body; primary sites were the thymus, the thorax, the retroperitoneal area, the gonads, and the epididymal fat pads. It was quickly demonstrated that prothrombin times in animals fed diets containing irradiated beef were elevated and that the addition of small amounts of vitamin K_1 or vitamin K_3 returned prothrombin times to normal. Other laboratories received contracts from the Army to assist in the elucidation of these problems. Among these were the Army Medical Research and Nutrition Laboratory at Denver, Richardson's group at Texas A & M, Reber at the University of Illinois, Mellette at Richmond, Virginia, and our group at St. Louis University.

Table 1 shows some of the problems which have been studied by these groups. In fairly quick succession, it was shown that there was a very remarkable difference in susceptibility of different strains of rats to hemorrhage, that female rats were much more resistant to hemorrhage than males (28, 29), and that irradiation was not necessary for diets to cause nutritional hypoprothrombinemia. Johnson's group first showed that soy protein was more hemorrhagenic than irradiated beef (21), and that a straight chain aldehyde, decanal, lowers prothrombin (17). In an extension of the older observations of Butt and Snell (7), and of Greaves and Schmidt (13), that bile is necessary for the absorption of vitamin K, the Denver group showed that a few milligrams of free bile acids added to the diet alleviated the hemorrhage and decreased its incidence (17). About this time, Barnes and Fiala developed a technique for the prevention of coprophagy by tail cups, and showed that male rats suffered hypoprothrombinemia by the simple exclusion of 2-methyl-1,4-naphthoquinone from the diet (3, 4).

The Illinois group used this technique and a circular tubular cage to prevent coprophagy, and found animals thus maintained suffered more severe K avitaminosis. In such noncoprophagic rats, they determined that the weanling male rat will have a normal prothrombin time at 4 weeks if the diet supplies about 1.5 μg of vitamin K_1 per day (17). Read et al. of Denver reported that rats maintained normally and fed irradiated beef need

[1] Preliminary reports of some of our work mentioned in this discussion have appeared (25–27, 31). These investigations were supported in part by Contract No. DA-49-007-MD-996 with the Offices of the Quartermaster and Surgeon General, Department of the Army. The opinions expressed herein are those of the author, and not necessarily those of the Department of the Army.

[2] By general agreement of investigators, participants were assigned certain topics and were permitted to draw freely upon the work of other contractors.

about 4 μg of vitamin K_3 daily; while the addition of 83 mg of bile acids per day lowers this requirement to 2 μg (17, 28).

Using the chick assay for vitamin K, Johnson's group has shown that vitamin K activity equivalent to 8–19 μg of vitamin K_3 is present per gram of rat cecal contents. Thus, only a small amount of coprophagy would be necessary in order to meet the animal's daily requirement of 1.5–2 μg. As to the differences between strains, the Holtzman, Sprague-Dawley, and Charles River CD strains are all readily susceptible to hemorrhage; while the Texas A & M and St. Louis University strains are more resistant (28). In part, this may be due to the degree of coprophagy practiced by the different strains, and, in part, it may be related to the nutritional status of the mother (Richardson, unpublished observations). It should be pointed out that critical experiments to ascertain the requirements of these various strains for vitamin K have not been performed.

The remarkable resistance of the female rat to nutritional hypoprothrombinemia has received attention (29). Mellette has shown that hormonal factors are involved. Castration of the male rat slows the onset of hemorrhage, while spaying females intensifies the incidence of hemorrhage. In addition, she has reported that older male rats (12–27 weeks of age) are much more prone to hemorrhage than 3–5-week-old rats. In general, administration of androgens intensifies, while estrogens ameliorate, hemorrhages (28).

In mentioning some of our contributions to the problem, I should like to thank my colleagues, Doctors Matschiner and Michael, for the privilege of discussing their work. Blood was drawn by cardiac puncture under light ether anesthesia, and prothrombin was determined by the method of Hjort, Rapaport, and Owren (16), which is reported to be more specific for prothrombin than other one-stage methods. Our base-line value of 100% prothrombin was determined on a large series of adult male rats raised on laboratory chow. Prothrombins above 85% are considered normal; the range of values as well as the number of animals with normal prothrombins are given in the tables. Our first data were obtained from animals fed experimental regimens for 8 weeks, while more recently we developed a superior assay which uses adult males fed for only 2 weeks. Comparison of prothrombin levels in young and old males reveals that many advantages accrue to the use of older males (Table 2).

At the time we began our studies, we knew our strain was resistant to vitamin K deficiency. Addition of irradiated beef[3] to our standard ration (9) led to little or no hypoprothrombinemia, while duplication of Johnson's diet exactly led to significant decrease in prothrombin. Examination of the dietary formulas led us to suspect the vitamin mineral supplement, since it was here that differences existed between Johnson's diet and ours. Our diets contained .2 international units (IU) of vitamin A per gram, while 1.5% cod liver oil furnishes 12–27 IU per gram, depending upon the potency of the cod liver oil.

TABLE 1. *Factors Influencing Nutritional Hypoprothrombinemia in the Rat*

Diet	Strain	Maternal diet
Beef	Age	Vitamin A
Soy protein	Sex	Vitamin A acid
Bile acids	Hormones	Oxidized lipids
Decanal		Protein
Coprophagy		Amino acids

TABLE 2. *Comparison of Old (13 Weeks) and Weanling Rats*

Diet*	Age	Time, week	Prothrombin % Normal		Normal Animals
			Avg.	Range	
JB	Old	1	51	29–78	0/10
		2	42	18–59	0/10
JB	Weanling	2	73	22–114	4/10
Casein	Old	1	74	52–91	1/10
		2	75	33–102	4/10
Casein	Weanling	2	96	32–121	9/10

* Diet JB contains 35% solids of beef irradiated at 5.58 megarad and cooked before incorporation. Composition: Beef-38, sucrose-19, cornstarch-35, salts 446-4, vitaminized cerelose-5, wheat germ oil-0.5, cod liver oil-1.5. Wet weight 153 g, solids 100. The casein diet contained in %: casein-21, salts 446-4, cornstarch-43, sucrose-26, vitaminized cerelose-5, corn oil-5, vitamin E acetate-.0088, viosterol-.027. Vitamin A acetate was added at 50 IU/g of diet.

It has been known for some time that massive amounts of vitamin A lead to hemorrhage and to hypoprothrombinemia, which are responsive to administration of vitamin K (18, 19, 43). However, levels of 2,000 to 15,000 IU of vitamin A per day were administered. Experiments designed to test the influence of vitamin A showed that animals fed diets containing irradiated beef had prothrombin levels which varied inversely with the amount of vitamin A added.

The vitamin was operative at minute levels in producing hemorrhage. An insignificant amount of vitamin A, as little as one-half of 1 IU/g of diet lowered prothrombin (Table 3) and higher levels progressively decreased the level of prothrombin without apparently affecting growth. Vitamin A assays with the Carr-Price reagent showed that there was no liver storage of vitamin A at 0 and 0.5 IU/g, but detectable hepatic levels occurred after feeding 5 IU/g of diet. This is somewhat less than that recommended for optimal growth in the rat (33, 38). Contrasted to this is the effect of vitamin A in rats fed purified ration containing casein as a source of protein (Table 4). It should be noted that the animals receiving no additional vitamin A were in extremis from vitamin A deficiency at the time blood was drawn, and despite this their prothrombin was not severely depressed. Much more vitamin A is needed in the presence of

[3] The ground beef used in these studies was purchased by the Office of the Quartermaster General and irradiated at 5.58 megarad by gamma rays at the Savannah River or Arco installations.

TABLE 3. *Effects of Adding Vitamin A Acetate to Diets Containing Irradiated Beef**

Vit. A added IU/g	Body Weight, g		8 week Prothrombin % of Normal	Normal Animals	Liver Vit. A IU/g
	Start	8 week			
0	41	260	101 (79–121)	8/10	0
0.5	44	328	75 (44–100)	5/10	0
5	44	324	39 (7–71)	0/9	140 (130–150)
50	46	287	40 (22–73)	0/9	968 (820–1,430)
500	46	250	10 (1–42)	0/8	18,300 (15,000–20,900)

* Diets as described by Johnson (17), except 1.5% cod liver oil replaced by an equal weight of corn oil containing the indicated amount of vitamin A acetate and 2.7 IU of vitamin D/g diet. Beef irradiated at 5.58 megarad.

TABLE 4. *Effects of Adding Vitamin A Acetate to A Purified Diet Containing Casein**

Vit. A added IU/g	Body Weight, g		8 week Prothrombin % of Normal	Normal Animals	Liver Vit. A IU/g
	Start	8 week			
0	48	213	85 (69–107)	4/10	0
0.5	44	265	96 (69–116)	8/10	0
5	48	287	88 (44–119)	7/10	78 (66–94)
20	44	251	83 (46–115)	5/10	470 (390–540)
50	45	279	66 (6–119)	4/10	1,030 (840–1,300)
500	44	238	15 (8–36)	0/10	13,400 (10,000–21,700)
500†	41	221	106 (93–117)	10/10	12,300 (10,100–15,600)

* Composition of diet in %: Casein-21, salts 446-4, cornstarch-43, glucose-22, vitaminized glucose-5, corn oil-5, vitamin E acetate-.0083 and viosterol-.027. Vitamin supplements do not contain added vitamin K. Composition of salt and vitamin mixtures is given in (17). † 2 μg of vitamin K diphosphate/g diet.

casein to produce similar depression of prothrombin (25, 26).

As an aside at this point, we were interested in other substances possessing vitamin A activity. Encouraged by Professor R. A. Morton of Liverpool, who visited our laboratories last Fall, we investigated a synthetic compound, vitamin A acid. It was synthesized originally by Arens and van Dorp, who reported that its sodium salt possessed biological activity equivalent to the natural vitamin (1, 42). It was also noted that the acid does not respond normally to the Carr-Price reagent (2), while Dowling and Wald have recently shown that it replaces the natural vitamin in all respects, except for maintenance of vision by the retinal rods (10, 11). Furthermore, this acid has not been detected in mammalian tissue, is not stored in the body, and is more toxic than the natural vitamin by other criteria. We tested the effects of the acid in casein diets free of added vitamins A and K, as shown in Table 5. Vitamin A acid is at least ten times more hemorrhagenic than the acetate of vitamin A. Similar toxicity by criteria other than hemorrhage was observed by Ames (unpublished, quoted by (15)). We are presenting these data at this time, because vitamin A acid is by far the most hemorrhagenic substance we have yet studied. Although the acid has not been detected in mammalian systems, Elder and Topper reported at these meetings that retinene is converted to vitamin A acid by enzymes found in several rabbit tissues (12).

Another fat soluble vitamin is implicated in this problem. Mellette has reported on a similar effect of vitamin E on prothrombin levels, although the effective amounts are much larger (28). A similar effect of α-tocopherylhydroquinone was noted by Mason and Rao (24) who reported that typical hemorrhages resulted in the usual loci; these did not respond to administration of vitamin E but were alleviated by vitamin K.

If we may now turn our attention upon another facet of the problem, reference has been made to the importance of dietary protein. Table 6 compares four of these, each added at the 21% level to a purified ration with vitamin A acetate at 50 IU/g. Casein and lactalbumin support prothrombin levels fairly well, while soy protein and zein do not. Furthermore, the method of preparation of the protein is critical (26). Johnson's group mentioned this last year (17) and in extending this observation we have extracted several proteins at room temperature with ethyl alcohol for 1 week. Table 7 shows that lipid solvent extraction increases the hemorrhagenicity of the protein.

Borden's Labco casein is not solvent extracted by the manufacturer, while General Biochemicals and Nutritional Biochemicals report that their caseins are extracted with hot ethanol. It is also apparent that soy protein is more damaging after extraction.

TABLE 5. *Comparison of Effects of Vitamin A Acetate and Vitamin A Acid in Adult Male Rats**

Diet Additions	Time, week	Weight Gain, g/day	Prothrombin % of Normal		Normal Animals
			Avg.	Range	
None	1		104	88–123	10/10
	2	1.91	98	47–131	8/10
Acetate 50 IU/g	1		87	73–102	5/10
	2	1.75	88	59–111	6/10
Acetate 250 IU/g	1		71	41–133	1/9
	2	1.65	51	24–117	1/10
Acid 5 IU/g	1		81	33–109	4/10
	2	2.04	61	39–85	1/10
Acid 50 IU/g	1		28	10–42	0/10
	2	1.57	11	5–23	0/10

* Diet as in Table 4.

As if the effects of protein were not enough, Malhotra and Reber reported upon the action of methionine in protecting against hemorrhage in rats fed irradiated beef (20). This may correlate with the earlier work of Carter and Warner, who showed that methionine and vitamin K_3 were necessary to maintain factor V (accelerator) activity in dogs (8). Table 8 presents our data corroborating the work of Reber, and showing growth restriction at the higher level of methionine. Another amino acid was also examined in view of the antagonism between tryptophane and vitamin K reported by Quagliariello et al. (35). As can be seen in Table 8, tryptophane had a hemorrhagenic action under these conditions.

Another phase of our investigation previously reported by Dr. Matschiner has dealt with the effects of addition of several lipids to the rations (27). We are indebted to Dr. Hsia of our laboratories for their oxidation. Table 9 shows the remarkable hemorrhagenic properties of squalene and oxidized squalene added to the casein ration containing 50 IU of vitamin A per gram of diet. This depression is readily combatted by the addition of 2 μg of dihydrovitamin K_1 diphosphate[4] per gram of food. Thus, we have added to the already numerous factors influencing nutritional hypoprothrombinemia in the rat. We have shown the extremely critical effect of vitamin A at low levels; the more pronounced effect of vitamin A acid; and the effect of certain oxidized lipids; and have pointed out the importance of preparation of certain proteins upon prothrombin level.

The surface of this problem has been scratched but not very deeply. There are many unanswered questions. Is intestinally synthesized vitamin K unavailable unless coprophagy is practiced? How are the K vitamins involved in the synthesis of prothrombin? What other clotting factors are also affected? How do the vitamins A and E and their derivatives, as well as other lipids, intensify hypoprothrombinemia? How do methionine and other amino acids enter the picture?

TABLE 6. *Examination of Hemorrhagenicity of Several Dietary Proteins in Adult Male Rats**

Diet	Time, week	Weight Gain, g/day	Prothrombin % of Normal		Normal Animals
			Avg.	Range	
Drackett (soy protein)	1		58	28–82	0/10
	2	3.17	40	12–72	0/10
Casein	1		87	73–102	5/10
	2	1.75	88	59–111	6/10
Lactalbumin	1		84	59–103	6/10
	2	3.16	88	74–107	5/10
Zein	1		64	32–99	1/10
	2	−1.90	63	36–84	0/10

* Diet as in Table 4; 21% protein, vitamin A acetate at 50 IU/g.

TABLE 7. *Effect of Extraction by Ethanol on Hemorrhagenicity of Dietary Protein**

Diet	Time, week	Weight Gain, g/day	Prothrombin % of Normal		Normal Animals
			Avg.	Range	
Labco casein (Borden's)	1		87	73–102	5/10
	2	1.75	88	59–111	6/10
Labco casein (extracted)	1		74	37–108	4/10
	2	2.15	59	18–88	1/10
Drackett (soy protein)	1		58	28–82	0/10
	2	3.17	40	12–72	0/10
Drackett (extracted)	1		31	14–62	0/10
	2	1.00	26	13–47	0/9
GBI casein	1		53	34–73	0/10
	2	2.64	46	33–74	0/10
NBC casein	1		47	24–99	1/10
	2	1.50	47	18–84	0/10

* Diet as in Table 4; 21% protein, vitamin A at 50 IU/g.

TABLE 8. *Effect of Supplemental Amino Acids on Blood Clotting of Adult Male Rats*

Diet*	Time, week	Weight Gain, g/day	Prothrombin % of Normal		Normal Animals
			Avg.	Range	
Soy protein	1		58	28–82	0/10
	2	3.17	40	12–72	0/10
Soy protein + 0.5% *d,l*-methionine	1		43	25–58	0/10
	2	2.16	37	17–72	0/10
Soy protein + 2.0% *d,l*-methionine	1		60	33–87	1/10
	2	0.62	62	26–91	1/10
Casein†	1		87	73–102	5/10
	2	1.75	88	59–111	6/10
Casein + 0.5% *l*-tryptophane	1		77	30–120	4/10
	2	1.21	53	24–105	2/10

* Composition same as in Table 4, except soy protein replaced casein. Vitamin A acetate added at 50 IU/g. † Data from Table 4.

TABLE 9. *Effects of Squalene and Oxidized Squalene on Prothrombin Level of Adult Male Rats*

Diet* Additions	Time, week	Weight Gain, g/day	Prothrombin % of Normal		Normal Animals
			Avg.	Range	
None	1		87	73–102	5/10
	2	1.75	88	59–111	6/10
0.5% squalene	1		51	35–88	1/10
	2	2.72	24	8–57	0/10
0.5% squalene + K_1 DP†	1		96	84–111	9/10
	2	1.43	82	62–115	4/10
0.5% oxidized squalene	1		22	13–30	0/10
	2	1.92	10	5–18	0/10
0.5% oxidized squalene + K_1 DP†	1		106	94–123	10/10
	2	2.83	101	88–111	10/10

* Diet as in Table 4 containing 50 IU vitamin A acetate/g diet. † K_1 DP is dihydrovitamin K_1 diphosphate at 2.5 μg/g diet.

[4] We are indebted to Dr. N. S. Ritter of Merck, Sharp and Dohme for a generous gift of dihydrovitamin K_1 diphosphate.

The other portion of my paper deals with the metabolism of the K vitamins. Several years ago Richert administered 300 mg of 2-methyl-1,4-naphthoquinone to rabbits and isolated the ethereal sulfate of the hydroquinone as a urinary metabolite (37). Later, the Canadian group of Jaques, Taylor et al. compared the metabolism of C^{14}-labeled K_1 and K_3 and, in addition to confirming Richert's work, observed that most of the radioactivity in the bile of rats with bile fistulas was altered. Only 1.5% of the C^{14} in bile was in unchanged vitamin K_1 (32, 39–41). Most of the early work measured distribution or metabolism after the administration of massive amounts, and to date no investigator has traced the metabolism of physiological amounts, i.e., 2–4 μg of vitamin K_1, through the rat or other experimental animal.

Among the various approaches to the problem are the elegant techniques of Martius et al. In 1955, he reported on the feeding of 80 μg/day of C^{14}-labeled 2-methyl-1,4-naphthohydroquinone diacetate for periods up to 70 days. The chickens were sacrificed, and heart, liver, and kidney were removed, homogenized and separated into subcellular fractions. Martius found two to seven times as much radioactivity in the mitochondria of these organs as in the nuclear fractions. Inquiry into the nature of the C^{14} was conducted by partition of the radiocarbon fractions between immiscible organic solvents. The partition coefficients of the metabolites did not resemble that of the substrate or of vitamin K_3 but were nearly identical to that of vitamin K_1 (22). The following year, Greene, Søndergaard, and Dam found by chick assay that activity equal to 71 μg of vitamin K_3 per 100 grams of fresh beef liver was present in hepatic mitochondria (14).

Martius then gave labeled menadione to chicks and rats and identified by countercurrent distribution 2-methyl-3-geranylgeranyl-1,4-naphthoquinone [$K_{2(20)}$]. This report in 1959 included data which showed that homogenates of chicken liver could make $K_{2(20)}$ from menadione and mevalonic acid (23).

In a follow-up study in 1960, Billeter and Martius gave chickens .355 mg of a mixture of labeled vitamins K_1: H^3 in the 2-methyl and C^{14} in the 1′, 2′ carbons of the phytyl side chain (6). After 6 hr, the animals were sacrificed and heart, skeletal muscle, liver, and kidney examined for H^3 and C^{14} labels. In the heart, ⅔ of the labeled vitamin K_1 had lost the carbon labeled phytyl group, which was replaced by the geranylgeranyl side chain giving $K_{2(20)}$. $K_{2(20)}$ was the major form found in muscle and kidney, while in liver most, if not all, of the label was present in unchanged K_1. They observed that not only did the pigeon convert more K_1 to $K_{2(20)}$ than the chicken, but the rat produced little or no detectable $K_{2(20)}$. In addition, they identified by countercurrent techniques the labeled phytol split from the substrate (6). Neither these studies, nor those dealing with the role of vitamin K in oxidative phosphorylation, have been correlated with the primary function of this vitamin in stimulating the production of prothrombin. Further experimentation combining the approaches of Martius with others, such as those of Barnhart et al. (5) and Pool et al. (34), may be required to elucidate the metabolic role of vitamin K.

One of the problems facing investigators of both in vivo and in vitro studies has been that of solubility of the vitamin in physiological fluids. Certain aspects have been emphasized by recent work in our department. Dr. Michael has synthesized vitamin K_1 labeled in the 3′-methyl group with C^{14}. Preliminary studies on its metabolism have resulted in several interesting observations concerning its absorption (31). Administration of 200–1000 μg. quantities intraperitoneally to rats in either dibutyl succinate or 5% Tween 80 permitted very slow absorption only, and 25–45% could be recovered by washing the peritoneum 16–20 days later. Absorption from the bowel was similarly low, even if the vitamin were dissolved in rat bile before administration.

Thus, our studies emphasize the importance of studying vitamin K of very high specific activity to obviate problems in solubility and to give enough isotope for accurate detection without exceeding the nutritional requirements of the animal. The metabolites from any vitamin given in excess may not reflect truly the metabolism of the physiologically active portion. Since the rat grows well and has normal clotting on an intake of 2 μg of vitamin K/day, the presumption can be made that this amount is adequate for both prothrombin synthesis and electron transport. Whether these functions are subserved by the same molecules of the vitamin, and whether one pool is more labile than the other, have not been clearly demonstrated.

In summary, interest in the safety of irradiated foods has stimulated a great deal of activity which has resulted in many interesting observations. Serious doubt as to the primary absorption of vitamin K synthesized by the flora may be entertained. Some interrelationships between the fat-soluble vitamins and other lipids have been pointed out, although the mechanisms deserve further scrutiny. The day is at hand when labeled vitamin K may be traced in physiological amounts through the organism and further light thrown upon its multiple roles. With the multifaceted techniques being brought to bear, as shown by the other participants of this Symposium, and the work of Martius and of others, many of the mysteries surrounding vitamin K will be speedily solved.

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Vitamin K and other quinones as coenzymes in oxidative phosphorylation in bacterial systems[1]

ARNOLD F. BRODIE[2]

Department of Bacteriology and Immunology, Harvard Medical School, Boston, Massachusetts

CERTAIN QUINONES APPEAR TO PLAY a fundamental role in cellular metabolism. They are associated with the multienzyme complex of the respiratory chain and have been implicated as coenzymes in electron transport (6, 15, 18, 25, 34, 44, 45, 55, 62, 66) and in oxidative phosphorylation (9, 10, 37, 48). These lipid-soluble cofactors of the respiratory complex, like the water-soluble coenzymes, participate directly in the catalytic mechanism by undergoing cyclic oxidation and reduction (8, 34, 54–58). The unique chemical properties of the quinones have made it possible to dissociate them from the respiratory chain without disruption of the spatial orientation of enzymes and other coenzymes necessary for coupled activity. The quinone-reconstituted preparations, in general, have some or most of the properties of the original systems.

The quinone-depleted systems (Table 1) permit a closer examination of the role of these cofactors in cellular metabolism. In animals, plants, and bacteria, the quinones are associated with the subcellular structures which carry out energy metabolism. Depletion of the natural compound from these structures and from whole cells (40) has been achieved by maintaining animals on a K-deficient diet (48), by extraction with lipid solvents (22, 36, 44, 50), or by selective destruction with light at 360 mμ (1, 7, 9, 19, 29). Depletion of the bound quinone results in a loss of the ability to conduct electron transport and to generate ATP. Restoration of one or both of these activities can be accomplished by the addition of quinone; however, only with some systems is there a strict requirement for structural similarity between the added quinone and the natural compound.

Reconstitution of both oxidation and phosphorylation with the bacterial systems studied (9, 10, 41) specifically requires the addition of the natural bacterial quinone or a closely related homologue. A requirement for the natural mammalian quinone, coenzyme Q_{10}-ubiquinone[3] (27, 32, 49, 53), for restoration of electron transport has been demonstrated with lipid-extracted mammalian mitochondria (26, 32, 33); however, restoration of coupled activity with mitochondria from K-deficient animals (46, 48), or following irradiation with light at 257 or 360 mμ (1, 3, 29), requires the addition of vitamin K_1. Phosphorylation associated with the oxidation of substrate amounts of menadiol[4] has been observed with particles from *Azobacter vinelandii* (59), and with untreated mammalian mitochondria (24, 25), indicating that the reduced naphthoquinone can serve as an electron donor. The role of the naphthoquinones in mammalian oxidation remains obscure, since this type of quinone cannot be detected in mitochondria.

With chloroplasts, restoration of the Hill reaction (4) and of photophosphorylation (42), following petroleum ether or heptane extraction, has been demonstrated with the natural quinone (plastoquinone[5]), vitamin K_1, and menadione. Although certain unrelated quinones show some activity (2, 4) with extracted chloroplasts, maximal restoration occurs only with the natural compound. Photo-oxidation and reduction of the plastoquinone has been demonstrated with chloroplasts (26), indicating a direct relationship to the photosynthetic process.

SUBCELLULAR DISTRIBUTION OF QUINONES IN BACTERIA

Naphthoquinones of the vitamin K_1 and K_2 types, as well as benzoquinones, are widely distributed in micro-

[1] This work was supported by research grant E-2674 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

[2] Senior Research Fellow, Public Health Service.

[3] The coenzyme Q-ubiquinone class of quinones are 2,3-dimethoxy-5-methylbenzoquinones which contain unsaturated isoprenoid side chains of varying length in the 6-position. The most common natural quinone in mammalian mitochondria contains a side chain consisting of 10 isoprenoid units.

[4] Menadiol (2-methyl-1,4-naphthohydroquinone).

[5] The plastoquinones are 2,3-dimethyl substituted benzoquinones with an unsaturated isoprenoid side chain in the 6-position.

TABLE 1. *Quinone-Depleted Systems*

	Natural Quinone	Means of Depletion	Activity Lost on Depletion	Restoration	References
Bacterial *M. phlei*	K_1-like	Light (360 mμ)	Electron transport and Coupled phosphorylation	K_1	7–10, 16, 19
E. coli	$K_2(C_{45})$ Q_8	Isooctane and Light (360 mμ)	Coupled phosphorylation	$K_2(C_{45})$	39–41
Mammalian Mitochondria fragments	Q_{10}	Isooctane	Electron transport	Q_{10}	26, 27, 32, 34, 37, 51, 52
Mitochondria	Q_{10} K(?)	Diet	Coupled phosphorylation	K_1	46–48
		Light (254 or 360 mμ)	Coupled phosphorylation	K_1	1, 3, 29
Plants Sugar beet	Plastoquinone (Q_9)	Petroleum ether	Hill reaction	Plastoquinone (Q_9)	4
		Heptane	Photophosphorylation		42
Spinach leaves	K_1	None	Photophosphorylation	Menadione and K_5	2

TABLE 2. *Subcellular Distribution of Natural Quinones in M. phlei and E. coli*

Microorganism	Fraction	Natural Quinone	Concentration μmoles/mg Dry Wt.
M. phlei	Particles	K_1-like	3.5×10^{-3}
	Supernatant fraction	K_1-like	6.0×10^{-4}
E. coli	Large particles	Benzoquinone (Q_8)	2.6×10^{-3}
	Small particles	K_2 (C_{45})	1.4×10^{-4}
		Benzoquinone (Q_8)	2.7×10^{-3}
	Supernatant	None	

The cells were disrupted by sonic vibration for 4 min and the cell free extracts fractionated by the usual procedures (6, 42). The quinones were extracted with a mixture of isooctane-isopropanol (3:1), purified on Permutit (11) and assayed spectrophotometrically and by colorimetric procedures.

organisms. Certain bacteria (e.g., *Escherichia coli* and *Azotobacter vinelandii*) contain both benzo- and naphthoquinones and are referred to as mixed quinone systems. The concentration of quinones in a given species can vary and depends upon the cultural conditions, phase of growth, and especially on aerobiosis (39, 41, 43). Anaerobic growth of a facultative anaerobe, such as *E. coli*, results in a reduction of the concentration of both the benzo- and naphthoquinone. The shift in quinone concentration with decreased oxygen tensions and failure to detect quinones in strict anaerobes suggests an association of these compounds with oxidative metabolism.

The subcellular distribution of quinones has been examined in two different microorganisms: *Mycobacterium phlei*, a strict aerobe containing a naphthoquinone similar to vitamin K_1 (7, 12, 38); and *E. coli*, which contains a benzoquinone (C_{40}) and a vitamin K_2-naphthoquinone (C_{45}). The distribution of these compounds in fractionated extracts is shown in Table 2. The quinones are localized in the subcellular particulate fraction, which is essential for oxidative phosphorylation. The particulate fractions from *E. coli* have different patterns of quinone distribution and different oxidative abilities. The large particulate fraction appears to contain most of the succinoxidase activity, while the small particulate fraction contains the DPN-linked terminal respiratory enzymes (41). Although some quinone can be detected in the supernatant fraction, the amount varies and appears to depend upon the degree of disruption of whole cells and upon fractionation procedures.

The small particulate fraction from *E. coli*, as well as the particulate fraction from *M. phlei*, represents a highly organized structural unit which is biochemically analogous to mitochondria (17). This fraction from *M. phlei* contains dehydrogenases (17), bound DPN (17, 63), flavins, the naphthoquinone (39), and cytochrome components *c*, *b*, and *a* (17). The molar ratio of naphthoquinone to cytochrome *b* is about 12:1 and the concensration with respect to the other electron carriers is tufficient to account for a coenzymatic role in electron transport. The natural naphthoquinone undergoes cyclic oxidation and reduction and appears to lie on a main pathway of electron transport, since following its de-

struction by irradiation it is required for reduction of the terminal respiratory pigments (19, 61).

ROLE OF QUINONES IN RESPIRATORY CHAIN PHOSPHORYLATION

Coupled phosphorylation with fractionated extracts of *M. phlei* requires the particulate fraction and soluble components found in the supernatant fraction (6, 15, 16). Evidence implicating the naphthoquinones in oxidative phosphorylation with bacterial systems was first obtained from an analysis of the role played by the fractionated soluble components and from the effects of uncoupling agents such as Dicumarol and lapachol (10, 14, 15).

The supernatant fraction contains at least two active protein components; one is necessary for oxidation, and the other is required for phosphorylation (6, 15). Attempts to resolve these two components have been unsuccessful; however, an association of menadione reductase with the supernatant components was observed following fractionation (6). Preparations capable of reconstituting coupled phosphorylation contain this activity. Experiments designed to shed some light on the role of the flavins in the coupled process also provided evidence for the participation of the naphthoquinones. Removal of the flavins from the supernatant fraction by treatment with acid ammonium sulfate resulted in the loss of ability by this fraction to restore activity to particles, (Table 3). Partial restoration of oxidation alone was achieved by the addition of either flavin adenine dinucleotide (FAD), or riboflavin phosphate (FMN). Restoration of coupled phosphorylation occurred only with the addition of a mixture of FAD and vitamin K_1.

The fractionated bacterial system has at least two demonstrable pathways of electron transport, which can be distinguished by their sensitivity to inhibitors and by their association with coupled phosphorylation. The nonphosphorylative pathway is FMN-linked, and is sensitive to low concentrations of parachloromercuribenzoate (pCMB) and is insensitive to cyanide; whereas the FAD-vitamin K_1 pathway, which is associated with

TABLE 3. *Flavin and Quinone Requirement Following Fractionation of Supernatant Components*

Systems	Additions	Oxygen (μatoms)	ΔPi (μmoles)	P/O
P + TS	None	0.0	0.0	0.0
P + TS	FAD	2.26	0.4	0.18
P + TS	FMN	1.28	0.0	0.0
P + TS	Vit K_1	0.0	0.0	0.0
P + TS	FAD + K_1	2.40	1.6	0.67
P + TS	FMN + K_1	1.58	0.0	0.0

The system consisted of 0.4 ml of particulate fraction (P) containing 22 mg of protein per ml, 0.3 ml of acid-ammonium sulfate fractionated supernatant material (TS) containing 14.8 mg of protein per ml, 20 μmoles malate, 10 μmoles Pi, 2.5 μmoles ADP, 1 mg of yeast hexokinase, 20 μmoles glucose, 15 μmoles $MgCl_2$, 25 μmoles KF and H_2O to a volume of 1.3 ml. In addition the vessels indicated contained 100 μg FAD, 500 μg FMN, and 6.7 μmoles of vitamin K_1 suspended directly in the treated supernatant fraction. Brodie (6).

TABLE 4. *Effects of Light (360 mμ) on Natural Naphthoquinone and Oxidative Phosphorylation*

Treatment	Natural Naphthoquinone, μmoles/mg protein	Additions	Oxidation (μatoms)	ΔPi (μmoles)	P/O
None	2×10^{-3}	None	5.4	6.3	1.17
		Vit. K_1	6.2	6.6	1.06
Irradiation (360 mμ)	None	None	0.0	0.0	0.0
		Vit. K_1	5.2	5.1	0.98
		M. phlei Naphthoquinone	3.4	2.5	0.74

The system consisted of 0.3 ml of irradiated *M. phlei* extract containing 24.8 mg protein per ml, 20 μmoles malate, 2.5 μmoles AMP, 20 μmoles glucose, 1 mg of yeast hexokinase, 15 μmoles $MgCl_2$, 25 μmoles KF and H_2O to a volume of 1.3 ml. The vessels indicated contained 6 μmoles vitamin K_1 or 2.2 μmoles of the purified natural naphthoquinone isolated (12) from *M. phlei*. The reaction was carried out at 30° for 7 min. The naphthoquinone concentration before and after irradiation was determined spectrophotometrically (11, 39).

phosphorylation, as well as coupled activity with unfractionated extracts, are both insensitive to low concentrations of the SH-inhibitor and are sensitive to cyanide (9, 14).

Further evidence for the participation of a naphthoquinone in oxidative phosphorylation was obtained by taking advantage of the sensitivity of quinones to light (7, 9, 10, 19). This method provided a means of destroying the natural particle-bound naphthoquinone without dissociating the enzymes involved in oxidative phosphorylation. Treatment of the bacterial extracts with light at 360 mμ, results in a destruction of the natural naphthoquinone and in a loss of the ability to conduct oxidative phosphorylation (Table 4). Complete restoration of both oxidation and phosphorylation requires the addition of the natural naphthoquinone from *M. phlei* or vitamin K_1. The kinetics of light inactivation (254 and 360 mμ) have been described in detail (7, 8, 10). Following irradiation both oxidation and phosphorylation are dependent upon the concentration of vitamin K_1 (10). The coupled activity with the naphthoquinone-restored system is similar to that observed with untreated preparations, since the activity is sensitive to DNP and cyanide and is insensitive to pCMB (9).

Coupled phosphorylation with the K-reconstituted system occurs only with DPN-linked substrates. Succinoxidase activity is destroyed following light-treatment. This activity is not restored by the addition of vitamin K_1, coenzyme Q, or α-tocopherol. Thus the succinoxidase pathway in *M. phlei* contains another light-sensitive component which has not been identified. Although coupled phosphorylation with the irradiated bacterial and mammalian systems (1, 3, 29) requires vitamin K_1 for restoration, this reactivation appears to be different in the two systems. Reactivation by vitamin K_1 with the irradiated mammalian system occurs with both the

TABLE 5. *Specificity of Reactived Light-Exposed System*

Naphthoquinone		Activity Restored
R_2	R_3	
CH_3	$-CH_2-CH=C(CH_3)-CH_3$	Oxidation and Phosphorylation
	$-CH_2-CH=C(CH_3)-(CH_2-CH_2-CH_2-CH(CH_3))_n-CH_3$, n = (1 to 4)	
CH_3	$-CH_2-CH_2-CH(CH_3)-(CH_2-CH_2-CH(CH_3))_3-CH_3$	Oxidation (cyanide sensitive pathway)
OH	$-CH_2-CH=C(CH_3)-CH_3$	
OH	$-CH_2-CH=C(CH_2OH)-CH_3$	
CH_3	H	Oxidation (pCMB sensitive pathway)
CH_3	OH	

succinoxidase and DPN-linked pathways and the relationship of this naphthoquinone to the natural mammalian quinone is obscure.

SPECIFICITY OF REACTIVATION

The light-treated bacterial system exhibits a specific dependence on vitamin K_1 or closely related homologues. The structural requirements for restoration of both oxidation and phosphorylation are listed in Table 5. Compounds which are active contain a methyl group in the C-2 position and an unsaturated side chain of at least 5 carbon atoms in the C-3 position (7, 8, 10, 56).

Restoration of coupled activity is also dependent upon the nature and length of the side chain (7, 10). The naphthoquinones of the vitamin K_1 series with a side chain of 5–30 carbon atoms are capable of restoring both activities, vitamin K_1 (C_{20}) being the most active. Some activity was observed with the C_{10} and C_{15} homologues of the vitamin K_2 series; however, they were not as effective as vitamin K_1. The other members of the K_2-series were completely inactive. The natural naphthoquinone from *M. phlei* was found to be the most active quinone tested.

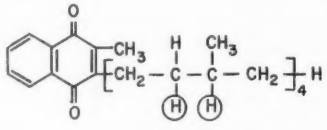
A number of K-like analogues with substitutions in the C_2 or C_3 position are capable of restoring only oxidation (Table 5). These compounds can be subdivided into two groups: those which, like menadione, transfer electrons directly to oxygen and are sensitive to pCMB; and those, like lapachol and dihydrophytyl[6] vitamin K_1, which transfer electrons by a pathway similar to that observed with vitamin K_1. Analogues of the latter group have been useful for demonstrating another phosphorylative site which occurs before the naphthoquinone in the respiratory chain (8, 10).

It is of interest to note that only naphthoquinones which can cyclize and form a chroman ring are capable of restoring oxidative phosphorylation (8, 56, 58). Saturation of the phytyl side chain of vitamin K_1 has a marked influence on phosphorylation (Table 6). Substitution of the saturated compound (dihydrophytyl vitamin K_1) for vitamin K_1 results in the restoration of only oxidation. This compound also acts as an uncoupling agent when added to non-irradiated preparations. Dallam and Taylor (30) have also tested the ability of the dihydrophytyl derivative to restore activity to irradiated mitochondria, and found it completely inactive. It would appear from the results with the bacterial system that cyclization is necessary for restoration of phosphorylation.

RELATIONSHIP OF NAPHTHOQUINONE TO PHOSPHORYLATION

Restoration of phosphorylation by vitamin K_1, and the fact that certain analogues lack this property while still able to function in the same electron transport pathway (Table 5), suggest the direct participation of vitamin K in phosphorylation. Additional evidence for a dual role for the naphthoquinone in oxidative phosphorylation has been obtained by examining the effects of analogues on the K-reconstituted system. Uncoupling agents which are structurally similar to vitamin K_1 (Dicumarol and lapachol) were found to be competitive inhibitors of phosphorylation with respect to vitamin K_1 (Fig. 1). The importance of the methyl group in

TABLE 6. *Effects of Saturation of the Side Chain of Vitamin K_1 on Oxidative Phosphorylation*



2,3-dihydrophytyl (K_1)

System	Additions	Oxidation μ atoms	Δ Pi μ moles	P/O
P + S	none	4.8	2.1	0.44
	K_1	5.08	2.4	0.47
	2,3-dihydrophytyl (K_1)	4.38	0.5	0.11
Irradiated P+S	none	0.10	0.0	0.0
	K_1	3.84	2.6	0.68
	2,3-dihydrophytyl (K_1)	2.12	0.0	0.0

The system was similar to that described in Table 4. In addition the vessels contained synthetic dihydrophytyl vitamin K_1 (6 μmoles).

[6] Dihydrophytyl vitamin K_1 (2-methyl-3-(2,3-dihydrophytyl)-1,4-napthoquinone).

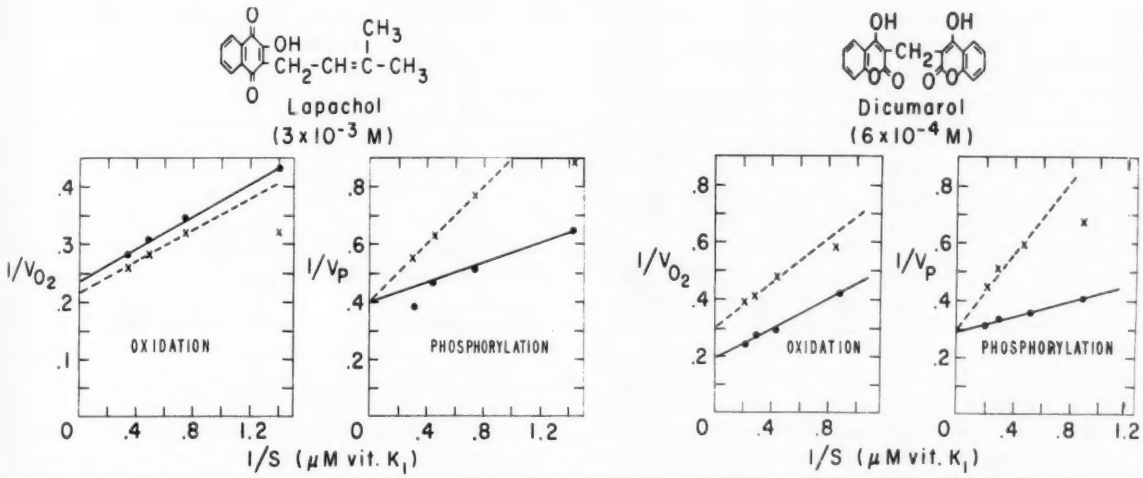


FIG. 1. Competitive inhibition by lapachol and Dicumarol of K-reconstituted phosphorylation. Brodie and Ballantine (10).

the C-2 position of the naphthoquinone nucleus is evident from the fact that lapachol differs from the first of the K_1 homologues, which restores oxidative phosphorylation, by the substitution of a hydroxyl group in that position. Uncoupling agents, which are not related to vitamin K_1 (DNP and thyroxine), were found to be noncompetitive inhibitors of phosphorylation with respect to vitamin K_1 (10).

Chemical evidence for the formation of metaphosphate and ADP from the oxidation of mono- and diphosphate esters of menadiol has been obtained by Clark et al. (21, 22) and Wieland and Patterman (65). To test the hypothesis that the naphthoquinones are directly involved in the phosphorylative events, certain model phosphorylated compounds of menadiol were prepared and tested for their ability to form ATP with the bacterial system (11). Oxidation of monoethyl menadiol monophosphate by the bacterial system requires a preincubation period, followed by the addition of phenazine methosulfate (Fig. 2). Maximal oxidation was observed when the reaction mixture included a phosphate acceptor system. The addition of Zwischenferment and TPN, following removal of the model compound, resulted in an increased oxidation with the complete system, which contained the phosphate acceptor system (hexokinase, ADP, and glucose). The additional oxidation which resulted from glucose-6-phosphate formed during the initial reaction was taken as an index of ATP formation. ATP formed from the oxidation of the model compound was also identified chromatographically and spectrophotometrically. The formation of ATP was found to be dependent upon the concentration of the model compound and was inhibited by DNP (11).

The chemical and biological experiments with model compounds suggest a means by which ATP can be formed from the oxidation of a naphthohydroquinone. However, it should be emphasized that neither the model compounds nor menadione can restore oxidative

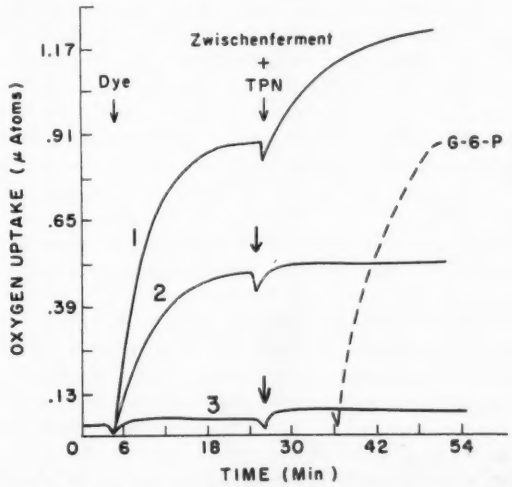


FIG. 2. Formation of G-6-P from menadiol phosphate oxidation. The complete system (*1*) consisted of 0.3 ml of *M. phlei* particles (20 mg protein per ml), 0.6 ml of the supernatant fraction (33 mg protein per ml), 10 μmoles of monoethoxymenadiol monophosphate, 2.5 μmoles ADP, 20 μmoles glucose and 1 mg of yeast hexokinase. *Reaction 2* lacked the phosphate acceptor system and *reaction 3* lacked the model compound. After incubation for 5 min the reaction was started by the addition of phenazine methosulfate (200 μg). The reaction was stopped by precipitation of the model compound with excess Mg^{++}, the precipitate removed, and the oxidation of G-6-P initiated by the addition of Zwischenferment and 100 μg of TPN to the systems.

phosphorylation with the light-treated system. These compounds are useful, nevertheless, for studying the physical and chemical properties of possible quinol intermediates.

INTERMEDIATES FORMED DURING ENZYMATIC REDUCTION OF VITAMIN K_1

The naphthohydroquinones are extremely unstable in air and must be trapped for analysis. These com-

pounds can be stabilized by substitution of the quinol hydrogens with a variety of chemical groups (acetate, benzoate, or phosphate). Analysis of various synthetic quinol derivatives (8, 18) revealed differences in the UV and fluorescence spectra between the dihydro-derivatives, and between the oxidized and reduced compounds, which could be used for identification. The dihydro-substituted quinols (phosphate and acetate) have a maximum absorption peak at 232 mμ, but can be differentiated by their characteristic absorption in the higher wave lengths (290–340 mμ). The alkoxy derivatives of menadiol, naphthochroman (naphthotocopherol), and dihydro vitamin K_1 have a maximum absorption peak at 245 mμ. Although the two compounds of particular interest, the chroman and dihydro-derivative of vitamin K_1, have similar absorption characteristics, they can be differentiated, following acetylation, by the difference in the absorption and fluorescence spectra of their acetyl derivatives, and by the Furter-Meyer reaction (31) (Table 7).

The *M. phlei* system, which can be made dependent upon vitamin K_1 for restoration of oxidative phosphorylation, has been used to investigate the intermediates formed during reduction of vitamin K_1, thus providing a means of studying the relationship between the intermediates formed and the coupled process. Two reduced naphthoquinone intermediates were found to accumulate when extracts capable of oxidative phosphorylation were incubated anaerobically with substrate amounts of vitamin K_1 and either DPNH or malate as electron donors (58). The reduced intermediates were trapped by acetylation and purified on Permutit. The acetyl derivatives were identified by chromatographic and spectrophotometric procedures. One compound was found to be identical to synthetic vitamin K_1 diacetate (max. 232 mμ, with minor peaks at 270, 278, 288 and 320 mμ) (Fig. 3*B*). The other acetylated derivative, which was formed enzymatically, was similar to synthetic monoacetate of the naphthochroman of vitamin K_1 (max. 245 mμ with minor peaks at 295, 301–308, 314, 322 and 328 mμ), (Fig. 3*A*). The enzymatically formed compound differs by the absence of the minor peaks at 314 and 328 mμ and by the presence of an additional peak at 338 mμ, (Fig. 3*A'*). The acetylated naphthochroman-like compound from the reaction mixture[7] gave a positive Furter-Meyer test providing further evidence for cyclization.

TABLE 7. *Differences Between the Naphthochroman and Dihydronaphthoquinone*

	Naphthochroman, mμ	Naphthohydroquinone, mμ
Absorption max. (isooctane)		
unacetylated	245	245*
acetylated	245	232
Activation (max.) acetylated	340	325
Fluorescence (max.) acetylated	400 (730†)	670
Furter Meyer test for cyclization	Positive	Negative

* Unstable. † Minor fluorescence peak.

Direct extraction with ether, in the absence of an agent to stabilize the reduced compounds which accumulate, results in the spontaneous oxidation of the dihydronaphthoquinone and provides a means of measuring the naphthoquinone-chromanol forming reaction (57). The results of direct ether extraction are shown in (Fig. 4). The spectrum obtained following extraction (o-time) is consistent with that of a mixture of synthetic naphthochroman and vitamin K_1. On standing, the spectrum of the ether extract was found to change gradually to one essentially identical with that of vitamin K_1. The rates of autooxidation of the chroman and formation of vitamin K_1 are shown in the upper half of Fig. 4. A difference spectrum, obtained by measuring the ether extract against a blank containing an equal concentration of vitamin K_1, was similar to that of synthetic naphthochroman. Since residual dihydroquinone can contribute to the 245 mμ absorption, an aliquot of the ether-extractable material was acetylated and the acetyl derivates examined. The ether-extractable material from the reaction mixture yielded only the naphthochroman.

The rate of formation of the chroman was found to be a function of the enzyme concentration, length of incubation, concentrations of vitamin K_1 and the electron donor, and of anaerobiosis (57). Although the formation of this compound was not dependent upon the presence of inorganic phosphate, more of the chroman was formed when phosphate was present. This compound was not formed with preparations which had lost the ability to couple phosphorylation to oxidation (57, 58).

Further evidence indicating a relationship between the naphthochroman and oxidative phosphorylation was obtained with the fractionated mycobacterial system. Since oxidative phosphorylation with the fractionated system is dependent upon both the particulate and supernatant fractions, these two fractions were tested for their ability to form the chroman. The particulate fraction alone cannot form this compound; however, small amounts are formed by the supernatant fraction. Maximal formation of this compound, like the coupled activity, requires the participation of both fractions (57, 58). Chroman formation is inhibited by Dicumarol (10^{-5} M), whereas DNP has little or no effect on chroman formation and causes an increase in the amount of dihydroquinone formed.

The specificity for restoration of oxidative phosphorylation, with respect to the 2,3-position of the naphthoquinone nucleus, has a direct relationship to the structural requirements for chroman formation. All of the naphthoquinones which are active in restoring both

[7] Further evidence for the chroman structure of the enzymatically formed compound has been recently obtained by comparison of the nuclear magnetic resonance spectra of the synthetic and enzymatically formed compound by Folkers and Trenner of Merck, Sharp and Dohme. The enzymatically formed compound differed from the synthetic chroman of vitamin K_1 by the absence of one of the methyl groups; this may account for the minor differences in the ultraviolet absorption spectra.

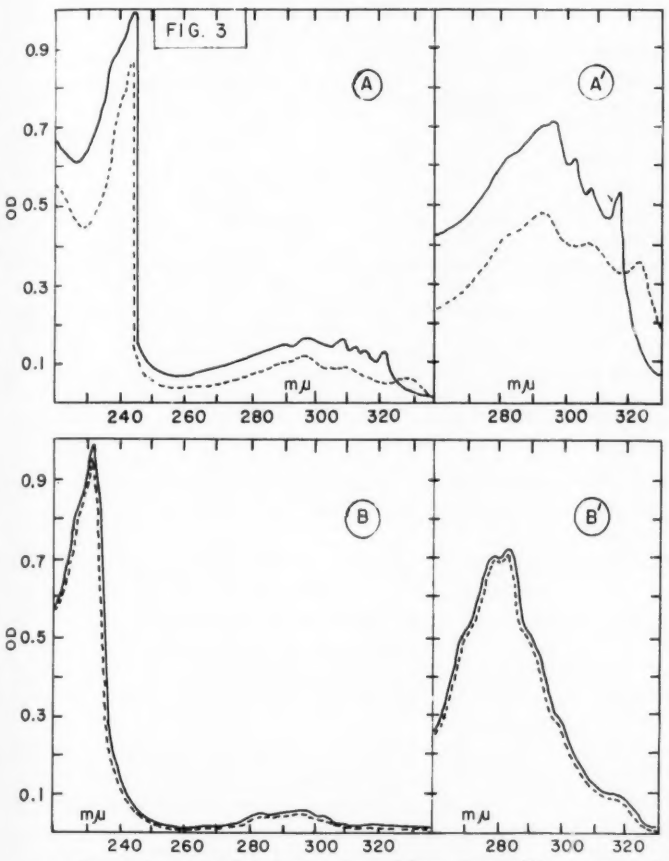


FIG. 3. Trapped acetylated derivatives formed from vitamin K_1 in anaerobic reaction. The system consisted of 8–10 ml of crude *M. phlei* extract (30 mg protein per ml), 200 μmoles of malate, 100 μmoles of vitamin K_1, 100 μmoles Pi, 50 μmoles $MgCl_2$, 50 μmoles KF. The reaction was carried out at room temperature under strictly anaerobic conditions for 2 hr and then the water removed by lyophilization. The dried material was acetylated, extracted with ether and fractionated on Permutit.

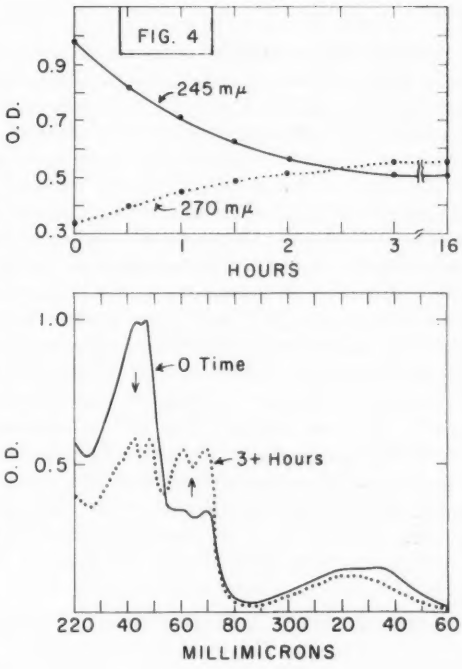


FIG. 4. Spectrum of ether extract of the anaerobic reaction mixture. The conditions were similar to those in Fig. 3. Following the removal of water, the dried material from the reaction mixture was directly extracted with ether.

oxidation and phosphorylation are structurally capable of forming the β-chroman ring (Table 8). Examination of the structure of analogues with substitutions in the 2,3-position, which are only capable of restoring oxidation, revealed that, with the exception of lapachol, all of the remaining analogues tested are unable to cyclize. Lapachol is theoretically capable of forming an α- or β-chroman. The analogues were also tested with the anaerobic system and the reduced derivatives isolated and analyzed (Table 8). Chroman formation did not occur with the analogues (56), which only restore oxidation to the light-treated system.

Phosphate appears to be associated with the fraction containing the naphthochroman[8] (8, 18, 56, 58). The ease of auto-oxidation of this lipid-soluble compound has afforded a means of determining whether it contained organically bound phosphate, since phosphate liberated during oxidation could be detected in the water phase. The rate of phosphate release into the aqueous phase was found to be equivalent to the rate of formation of vitamin K_1 from the chroman (58). Acceleration of the rate of destruction of the chroman by exposure to light at 360 mμ resulted in an acceleration of the rate of phosphate release. Phosphate was detected following cyclization with the natural *M. phlei* compound, vitamin K_1 (C_5) (2-methyl-3-(3-methyl-2-butenyl)-1,4-naphthoquinone) and with vitamin K_1 (Table 9). The amount of phosphate associated with the intermediate formed from vitamin K_1 (C_5) was less than that associated with K_1 (C_{20}). This homologue is also less effective than vitamin K_1 (C_{20}) in restoring oxidative phosphorylation. Phosphate was not detected with the hydroquinones formed from lapachol, menadione, or dihydrophytyl vitamin K_1.

[8] Further evidence for the existence of the chroman phosphate has been obtained following bromine oxidation of the isolated intermediate in acetic acid by the method of Clark et al. (21). Oxidation of quinol phosphates by this procedure results in the formation of acetyl phosphate, which can be trapped and detected as the hydroxamate. Acetyl phosphate (identified chromatographically as the hydroxamate), is formed by bromine oxidation in acetic acid of the enzymatically formed intermediate.

ENZYMATIC OXIDATION OF NAPHTHOCHROMAN

It was of interest to determine whether the enzymatically formed chroman could serve as an electron donor and generate ATP. The isolated intermediate formed from vitamin k_1 anaerobically was placed in the dry state in the main compartment of a Warburg vessel and fresh enzyme and ADP were added from the side arm (58). The intermediate served as an electron donor, and ATP was formed (Table 10). DNP (10^{-3}M) inhibited the formation of ATP but did not affect oxidation. Under anaerobic conditions, ATP was not formed, suggesting that oxidation is necessary for ATP formation. Dallam (28) has recently presented a scheme suggesting that phosphate transfer can occur in the absence of oxidation. Experiments designed to answer this question have shown that the reduced chroman intermediate must undergo oxidation before transfer of phosphate to ADP can occur. The chroman was allowed to form and accumulate in the usual manner; ADP, hexokinase and glucose were added to the anaerobic mixture for the last 10 min and the fate of the intermediate followed.

TABLE 8. *Correlation Between Chroman Formation from Various Quinones and Reconstitution of Oxidative Phosphorylation*

Compound	Ability to Reconstitute		Intermediate Isolated
	Oxidation	Phosphorylation	
Vitamin $K_1(C_{20})$	+	+	β-Naphthochromanol
Natural *M. phlei* Naphthoquinone	+	+	β-Naphthochromanol
Vitamin $K_1(C_5)$*	+	+	β-Naphthochromanol
Menadione	+	−	Naphthohydroquinone
Lapachol	+	−	Naphthohydroquinone
Dihydrophytyl Vitamin K_1	+	−	Naphthohydroquinone
Vitamin $K_2(C_{45})$	−	−	None†
Benzoquinone (Q_{10})	−	−	None†

* Vitamin $K_1(C_5)$, 2-methyl-3-(3-methyl-2-butenyl)-1,4-naphthoquinone. † Compound is not reduced by the *M. phlei* system.

TABLE 9. *Inorganic Phosphate Appearance During Chroman Oxidation*

Compound	ΔK (μmoles)	ΔPi Liberated (μmoles)	ΔPi/chroman
Vitamin $K_1(C_5)$	3.2	0.48	0.15
Vitamin $K_1(C_{20})$	2.5	2.1	0.85
M. phlei naphthoquinone	2.6	2.4	0.92
Vitamin $K_2(C_{35})$	0.0	0.0	—

The chromans formed enzymatically from the various quinones indicated were extracted with ether and immediately centrifuged. Water was added to the ether extract, the mixture shaken, and an aliquot of the water removed for phosphate analysis at 0 time and following the complete autooxidation of the chroman to the naphthoquinone (ΔK).

TABLE 10. *Distribution of P^{32} Following Enzymatic Oxidation of Isolated Intermediate*

Conditions Following Isolation	Oxidation, μatoms	P^{32} Distribution, cpm/μmole		
		ATP	ADP	AMP
Aerobic	2.6	292	28	28
Aerobic + DNP	1.6	0.0	2.8	2.5
Anaerobic		0.0	0.8	0.0

Conditions for formation of the chroman were similar to those described in Fig. 3 except that Pi^{32} (1118 cpm/μmole Pi) was added to the anaerobic reaction mixture. The intermediate formed was extracted with ether, immediately placed in Warburg vessels, and the ether removed by evaporated *in vacuo*. Following equilibration the contents of the side arm (0.5 ml of *M. phlei* extract (46.6 mg protein per ml), 5 μmoles ADP, 15 μmoles $MgCl_2$, and 25 μmoles KF) were added and the reaction carried out for 25 min at 30°. The reactions were terminated by the addition of TCA and the adenosine nucleotides purified by adsorption onto and elution from norit and separated as AMP, ADP and ATP on Dowex 1-chloride.

Although the chroman intermediate was formed before the addition of the acceptor system, this addition did not result in the disappearance of the chroman, or alter the amount of phosphate associated with the isolated intermediate. Experiments with model compounds (11, 21–23) also indicate that oxidation is necessary for ATP formation.

Since ATP is formed during the enzymatic oxidation of the reduced intermediate, it is essential to determine whether phosphorylation occurs at the quinol level of oxidation or at another site higher in the terminal respiratory pathway. The incorporation and distribution of P^{32} following the enzymatic oxidation of the isolated intermediate (formed in the presence of P^{32}-labeled inorganic phosphate), should provide evidence for the direct participation of the chromanol phosphate. An analysis of the adenine nucleotides (Table 10), revealed that the label was almost exclusively in the ATP; however, the specific activity of P^{32} incorporated into ATP (2–24%) during oxidation was lower than expected for a direct transfer of phosphate from a phosphorylated intermediate (18, 55, 58). Nevertheless, it seems unlikely that the phosphorylation is associated with oxidation at a higher level (the cytochromes), since phosphorylation at this level is lost during fractionation of the *M. phlei* extracts (6).

DISCUSSION

A number of mechanisms have been proposed for the formation of ATP from a phosphorylated quinol during oxidative phosphorylation. These schemes are based on possible chemical reactions or on experiments with model compounds (10, 11, 21–23). Three general types of reactions have been proposed and are shown in Fig. 5.

The first reaction shown was proposed by Wessels (64) and has been modified by Harrison (35), Clark et al. (22), Wieland and Patterman (65), and Brodie and

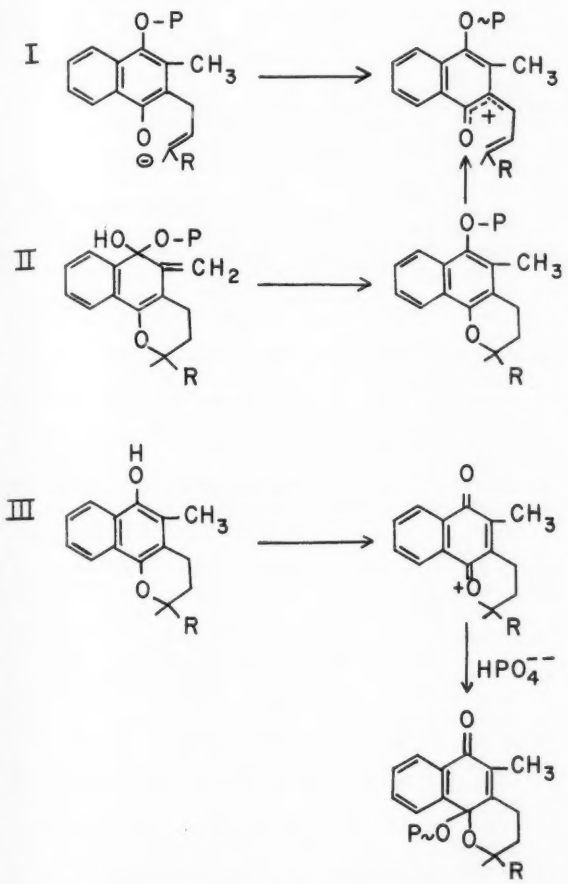


FIG. 5. Reactions proposed for formation of ATP from the oxidation of quinol phosphates.

Ballantine (10). Activation of the quinol phosphate in reaction 1 is accomplished by the removal of 2 electrons. The energy-rich enol phosphate ester formed during oxidation can react with ADP to form ATP. Experiments with model compounds indicate that this type of reaction is chemically and enzymatically feasible; however, the proposed schemes start at the quinol phosphate level and do not suggest a mechanism for its formation.

Reactions 2 and 3 involve cyclization between the carbonyl group of the naphthoquinone and the γ-position of the side chain. In scheme 2, proposed by Chmielewska (20), phosphate addition occurs following a charge separation at the 1-carbonyl position. Reduction results in the formation of chromanol phosphate, which must be oxidized as in scheme 1 before transfer of phosphate to ADP can occur. Scheme 3 has been proposed by Slater (60), Boyer (5), Clark and Todd (23), and, recently, by Dallam (28). In this scheme, the chroman formed during reduction is oxidized to yield a positively charged carbonyl group which in the presence of nucleophilic phosphate dianion, forms the chroman phosphate. This scheme differs from reaction 2 in that oxidation of the quinol phosphate is not necessary for ATP formation.

From the available biological evidence it is difficult to draw any conclusions regarding the mechanisms proposed, since little is known about the biological formation of quinol phosphate or the site on the quinone of phosphate addition. However, it is possible to make some generalizations. The quinones appear to act as "transformers," shuttling electrons between two coenzymes (DPN and the cytochromes) of the respiratory chain. Studies with analogues and dihydrophytyl vitamin K_1 suggest the direct participation of the quinone in the phosphorylative events, and indicate that cyclization to the chroman structure is necessary for participation of the naphthoquinone in phosphorylation. The enzymatic formation of a chroman intermediate from vitamin K_1 (a necessary component for restoration of phosphorylation with this system), and the parallelism between the conditions necessary for chroman formation and oxidative phosphorylation, further support this view. The data presented support a role for the chroman in electron transport; however, further evidence is necessary to establish the chroman phosphate as an intermediate in oxidative phosphorylation. Although the naphthoquinone-chromanol reaction has been studied only in bacterial systems, it is likely that a similar cyclic process of oxidation-reduction involving quinones occurs in animal and plant tissues.

The contributions of Dr. Percy J. Russell Jr., Miss Jane Ballantine, and Mrs. Eva Kashket are gratefully acknowledged.

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Role of vitamin K in electron transport[1]

WALTER D. WOSILAIT

Department of Pharmacology, New York State University, Downstate Medical Center, Brooklyn, New York

In previous studies (26) of biochemical reactions involving quinones, an enzyme was found which catalyzed the reduction of menadione (2-methyl-1,4-naphthoquinone) by the reduced pyridine nucleotides. This activity was tentatively designated pyridine nucleotide-menadione reductase, and was found to be widely distributed in bacteria, higher plants, and various mammalian tissues. Since menadione had not been reported to be present in tissues, it seemed possible that it might be serving as an analogue for the widely distributed vitamin K,[2] which also possesses the naphthoquinone nucleus. In view of the fact that vitamin K is found in organisms such as plants and bacteria, which do not possess clotting systems, as well as in mammalian tissues such as heart and kidney, which presumably are not involved in the synthesis of clotting factors, the question arose as to the role of vitamin K in these organisms and tissues.

The ease with which the quinones undergo oxidation and reduction, suggested the possibility that vitamin K might be involved in electron transport, and this possibility was supported by the finding of the above noted enzyme capable of reducing the naphthoquinone nucleus. In addition, phosphorylated derivatives of reduced menadione had been in clinical use, and it seemed possible that the enzymatically reduced product might also undergo phosphorylation and, thus, participate in oxidative phosphorylation.

In the early studies (26), vitamin K_1 had been tested as an electron acceptor in the form of an emulsion because of its insolubility in water. In this form, it did not appear to serve as an electron acceptor in place of menadione, but it was not certain that such emulsions were suitable for enzyme studies. In recent studies (22), various "solubilized" preparations of vitamin K_1 were tested with a menadione reductase purified from dog liver on the assumption that the naphthoquinone nucleus was the functional moiety in oxidation and reduction reactions, and it seemed possible that menadione reductase might also include vitamin K within its specificity. A procedure was finally developed with the use of BRIJ-35 (polyoxyethylene lauryl alcohol), which solubilized vitamin K_1 so that it substituted for menadione in the assay system. To date, these two activities have not been separated. Since vitamin K_1 is probably closer in structure to the natural electron acceptor than menadione, this activity will be referred to as vitamin K_1 reductase. The activity of the enzyme in homogenates was great enough so that it seemed possible it might play a role in respiration. In view of the fact that the enzyme was inhibited by various anticoagulants, the effect of the anticoagulants on the respiration of rat liver slices was also studied. These agents were found to depress respiration (23), and the relative order of potency on the slices was similar to the inhibitory effects on vitamin K_1 reductase.

In considering the fate of reduced vitamin K_1, one possibility considered was that it might, in turn, reduce a cytochrome such as cytochrome *c*. In an investigation of various cell fractions of rat liver, the mitochondrial fraction was found to catalyze the reduction of cytochrome *c* by reduced vitamin K_1 (25), thus regenerating the oxidized form of the vitamin. This report will be concerned with the reduction of vitamin K_1 by the reduced pyridine nucleotides, the subsequent reduction of cytochrome *c* by reduced vitamin K_1, and the possible participation of these systems in electron transport and respiration.

SOLUBILIZATION OF VITAMIN K_1

Vitamin K_1 is insoluble in aqueous media; and therefore, it could not be tested directly as a possible electron acceptor. In view of this, various approaches were tried to solubilize it, so that it could be tested in place of menadione, with a menadione reductase obtained from dog liver. Among the procedures tried were ultra sonic oscillation, binding to proteins, and solubilization with various detergents. Although a number of these preparations were visually clear, they did not substitute for menadione in the assay system; and as a result clarity was not a suitable criteria in itself. However, with the use of BRIJ-35, clear preparations were obtained, which substituted for menadione with the purified enzyme. These preparations will be referred to as solubilized vitamin K_1.

[1] These studies were supported in part by grant H-3055 from the Public Health Service.

[2] The term "vitamin K" used in this paper refers to a group of methyl-naphthoquinones which are necessary for the formation of clotting factors of higher animals in accord with the definition of Dam and Søndergaard (5), while "vitamin K_1" refers to 2-methyl-3-phytyl-1,4-naphthoquinone.

Since vitamin K_1 is destroyed by various types of treatment, the solubilized preparations were analyzed for possible decomposition resulting from the treatment. The following points indicated that vitamin K_1 had not been decomposed to an appreciable extent as a result of the treatment used to solubilize it, and such preparations appeared to be suitable for the present enzyme studies. The spectrum of the solubilized vitamin in the oxidized and chemically or enzymatically reduced form, was essentially the same as that of vitamin K_1 dissolved in 95% ethanol. These samples also provided the characteristic cobalt blue color of vitamin K_1 in the chemical determination described by Irrevere (11). Finally, the solubilized vitamin migrated in the same fashion as reference samples of vitamin K_1 when chromatographed. On the other hand, samples of the solubilized vitamin, which had been exposed to ultraviolet light, exhibited an altered spectrum, and also manifested an atypical color in the chemical determination.

Since the procedure used is not one commonly employed with substrates for enzyme studies, it was of interest to obtain some information concerning the means of solubilization. Detergents lower the surface tension of water, and this aspect was studied, but the amount of detergent required to solubilize the vitamin was several times that required to lower the surface tension to a low value, which suggested that this method was not the explanation. It was noted that 3.6% solutions of BRIJ-35 could solubilize limited amounts of the vitamin. The average molecular weight of BRIJ-35 was reported to be about 1215, while the micelle weight was about 48,800. A stoichiometric analysis of the amount of vitamin K_1 solubilized by the amount of detergent used, showed about a 1:1 relationship between the amount of vitamin solubilized and the micelle. Dialyzed samples of the solubilized vitamin showed a characteristic increase in volume without loss of the vitamin, which would support the view that it was in a micelle. Apparently the micelle was relatively stable, but evidently not completely so, since the solubilized vitamin still chromatographed the same as reference samples of vitamin K_1. Admittedly such a micelle is artificial, but it still may resemble that found in biological systems. Vitamin K was reported to be associated with the mitochondria (8), which have a high lipid content, and this would be a favorable site for the lipophilic side chains of vitamin K.

VITAMIN K_1 REDUCTASE

Although the enzyme has not been purified to a homogeneous state, so that definitive specificity studies could be carried out, the following information is available (22). Reduced di- and triphosphopyridine nucleotide served as electron donors in this reaction and were oxidized to DPN and TPN, respectively. The ratio of activity with these two substrates did not change appreciably over a 300-fold purification, or after attempted differential inactivation by changes in pH, by heat, or combinations of these. Benzoquinone, coenzyme Q_{10} (Ubiquinone), coenzyme Q_6, menadione, and vitamin K_1 served as electron acceptors, again with rather constant ratios of activity in the various fractions obtained. Samples of the enzyme tested from the early steps of purification did not exhibit a requirement for flavin in the assay system, but after chromatography on DEAE cellulose, such a requirement was exhibited, presumably as a result of the dissociation of a bound cofactor. The affinity of the enzyme for flavin adenine dinucleotide was about tenfold greater than for FMN. From dialysis studies FMN was found to be more readily dissociable from the enzyme than FAD. For example, samples of the purified enzyme were exposed to FAD or FMN and then dialyzed for up to 3 weeks. The samples which had been exposed to FMN showed a requirement for flavin in the assay system, but the FAD treated samples did not.

Reduced vitamin K_1 is rather readily oxidized, therefore, information concerning the enzymatically reduced product was carried by spectrophotometric procedures, rather than by isolation and characterization. To prevent oxidation of reduced vitamin K_1, it was necessary to carry out certain experiments under anaerobic conditions, and when DPNH was in stoichiometric excess of vitamin K_1 the amount of DPNH oxidized was equivalent to the amount of vitamin K_1 present. Thus, there was a 1:1 relationship between the amount of DPNH oxidized and the amount of vitamin K_1 present. In view of the stoichiometry, and the fact that benzoquinone was reduced to hydroquinone, it would seem likely that the naphthoquinones which serve as substrates are reduced to the corresponding naphthohydroquinones.

The spectrum of the oxidized and enzymatically reduced form of vitamin K_1 is presented in Fig. 1. This experiment was carried out under aerobic conditions, and the amount of DPNH and enzyme used was in excess, so that the vitamin was kept in the reduced form for the spectral analysis. The decrease in the 340 mμ region is mainly due to DPNH oxidation. Vitamin K_1 contributes to a certain extent in this region, causing an apparent shift in the peak absorption. The major changes which can be used to follow the reduction of vitamin K_1 are the decrease in absorption in the 270 mμ region and the increase in the 245 mμ region. Since menadione also served as an electron acceptor, it would appear that the phytyl group at the 3-position of vitamin K_1 is not necessarily directly involved. However, in the case of vitamin K, it is possible that there may be a subsequent conversion to a naphthotocopherol-like substance which involves the side chain (18).

In intracellular distribution studies, vitamin K_1 reductase was found mainly in the soluble fraction (Table 1). Since vitamin K is associated with the mitochondria, it might be expected that vitamin K_1 reductase would also be associated with the mitochondria, but this was not the case. The possibility that the activity was dissociated during the isolation procedure cannot be excluded. However, it might be involved in the oxidation of extramitochondrial pyridine nucleotides. Recently, Conover and Ernster reported the coupling of TPNH oxidation

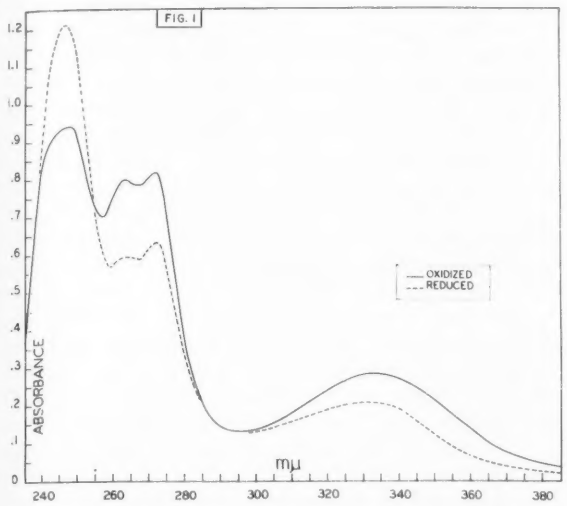

FIG. 1. Absorption spectra of oxidized and enzymatically reduced vitamin K_1. Samples of the reaction mixture containing phosphate buffer of pH 7.4, FAD, DPNH, and vitamin K_1 were transferred to citrate-versene buffer of pH 6.2 for examination in a Spectronic 505 recording spectrophotometer. The spectrum of the oxidized form was determined prior to enzyme addition while the spectrum of the reduced form was obtained 10 min after enzyme addition at which time the reaction appeared to be complete.

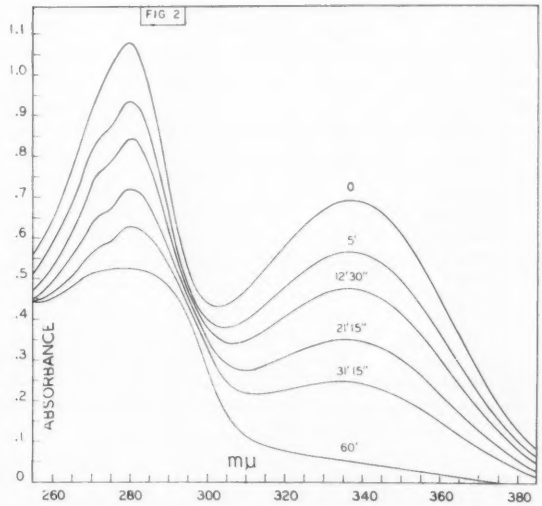

FIG. 2. Enzymatic reduction of coenzyme Q_{10}. The spectral changes occurring during the reduction of coenzyme Q_{10} by DPNH in phosphate buffer at pH 7.4 were measured at the indicated times in minutes.

with the mitochondria (4) by means of a soluble Dicumarol sensitive enzyme (6), but in these experiments menadione was used as an electron carrier.

Since benzoquinone served as an electron acceptor, it was of interest to test coenzyme Q_{10}, a naturally occurring tetrasubstituted benzoquinone, as a possible electron acceptor. Coenzyme Q_{10} is insoluble in water, however, using an adaptation of the procedure used to solubilize vitamin K_1, and preparations were obtained which were suitable for study. The spectral changes occurring during the reduction of coenzyme Q_{10} are shown in Fig. 2. The decrease in absorption at 275 mμ is mainly due to the reduction of coenzyme Q_{10}, while the decrease at 340 mμ is mainly due to the oxidation of DPNH. Similar spectral changes were observed when coenzyme Q_6 was used. When DPNH was in excess, there was a 1:1 stoichiometric relationship between the amount of DPNH oxidized and the amount of coenzyme Q_{10} present. The reduction of coenzyme Q_{10} by the purified enzyme was stimulated in a similar fashion by flavins as the reduction of vitamin K_1, and also was inhibited by the anticoagulants (24). To date, there has been no separation of this activity from vitamin K_1 reductase. In view of these results, it is possible that a benzoquinone derivative such as coenzyme Q, or a naphthoquinone derivative such as vitamin K or an as yet unidentified substance may be the natural electron acceptor.

In view of the antagonistic effects between Dicumarol and vitamin K in vivo, the effect of Dicumarol on vitamin K_1 reductase was studied, and it was found to be an inhibitory one. During purification, the sensitivity to Dicumarol was used to distinguish this activity from

TABLE 1. *Intracellular Distribution of Enzymes in Rat Liver*

Cell Fraction	Vitamin K_1 Reductase*	Vitamin K_1H_2 Cyt. *c* Reductase†
	μmole/min and g liver	
Mitochondria	0.36	0.09
Microsomes	0.09	0
Soluble	1.0	0

* Activity measured as DPNH oxidized in the standard vitamin K_1 reductase assay. † Activity based on cytochrome *c* reduction.

others, which also reduce menadione, but are not inhibited by Dicumarol. In view of the inhibition by Dicumarol, other anticoagulants were tested and found to inhibit the enzyme. The relative order of potency, based on the amount required for 50% inhibition, in decreasing order of potency, was as follows: bishydroxycoumarin (Dicumarol), phenindione, ethyl biscoumacetate (Tromexan), cumachlor (Indalition), warfarin, acenocoumarol (Sintrom), and salicylate. It may be noted that some of these agents, which are not members of the coumarin series but possess anticoagulant activity in vivo (e.g., phenindione and salicylate), also inhibited the enzyme. At least to this extent, there is a parallel between effects on the blood clotting system and the purified enzyme.

LIVER SLICE STUDIES

From the study of the inhibitory effects of Dicumarol on vitamin K_1 reductase, it was noted that the concentration required for inhibition was of a similar order of

TABLE 2. *Effect of Anticoagulants on the Respiration of Rat Liver (23)*

Agent	Concentration, M				
	10^{-5}	5×10^{-5}	10^{-4}	5×10^{-4}	10^{-3}
	%	%	%	%	%
Dicumarol	112	46	49	33	
Tromexan			115	37	32
Warfarin			96	80	78
Cumachlor			80	53	26
Sintrom			111	37	43
Phenindione			125	29	21
Salicylate				89	84

Respiration of the tissue samples was measured by standard manometric procedures. The values given, for the 30- to 60-min period, are expressed as per cent of respiration of control samples without anticoagulant.

magnitude as the concentration of Dicumarol reported in the livers of treated animals. For example, Green et al. (9), reported about 18–56 μg of Dicumarol per gram of rat liver and 55–78 μg per gram of liver in chickens which had been treated with Dicumarol. Assuming uniform distribution, this corresponds to about 5×10^{-5} M to 2×10^{-4} M, which is somewhat above that required to inhibit vitamin K_1 reductase. In addition, the amount of vitamin K_1 reductase in a homogenate was great enough that it seemed possible the enzyme might be involved in respiration (Table 1). For comparison, rat liver slices were found to consume oxygen at about 2–3 μmoles/min and gram wet weight at 25°. In view of the inhibition of vitamin K_1 reductase by the anticoagulants, and the possibility that this enzyme might be involved in respiration, experiments were carried out to determine the effect of these agents on the respiration of rat liver slices. Previously, Green et al. (7), reported that liver slices prepared from rats treated with cumachlor consumed oxygen at the same rate as control slices, but they did not report on studies in which tissue slices had been treated with anticoagulants in vitro. In the present studies, tissue samples were exposed in vitro to various amounts of anticoagulants.

Tissue samples were prepared with the use of a tissue chopper (16). Although these samples are not "slices" in the usual sense, they will be referred to as such. A comparison was made between the oxygen consumption of slices exposed to anticoagulant with control slices. After a period of exposure of the tissue to the drug, a depression of respiration was observed with each drug tested. For purposes of comparison, the depression observed during the 30- to 60-min time period is presented in Table 2. It is quite likely that these drugs enter the tissue at different rates and may be metabolized at different rates, which would influence the time of onset and extent of the depression. Furthermore, the intracellular concentration of each drug has not yet been determined, and as it is the tissue concentration which is critical, therefore a direct comparison of their potencies with their Ki for vitamin K_1 reductase cannot be made directly. However, if it is assumed that the amounts present are of a similar order of magnitude, a qualitative comparison shows that an agent which was a potent inhibitor of vitamin K_1 reductase, such as Dicumarol, was also a potent depressant of respiration. Salicylate was the least potent and the others were intermediate in effect in both cases.

A direct comparison of the in vitro effects with in vivo effects on the blood clotting system, again, would not be too meaningful because in vivo effects are further complicated by binding of the drug by blood proteins, metabolism, and excretion of the drug, all of which would affect the relative potencies. Since most of these agents also uncouple oxidative phosphorylation (15), the depressant effects cannot be attributed solely to inhibitory effects on vitamin K_1 reductase, and the possibility that some of the depression may be secondary to effects resulting from uncoupling must be considered. However, Green et al. (10), reported that the mitochondria obtained from treated rats contained about 9–13% of the Dicumarol in the homogenate, but the mitochondria were not uncoupled. They point out that these amounts may be low because some of the Dicumarol associated with the mitochondria may have been dissociated and removed during isolation of the mitochondria, thus decreasing its concentration.

In view of the fact that some blood proteins, e.g., albumin, bind Dicumarol (20), the possibility that tissue proteins might bind Dicumarol, causing a nonuniform distribution between the tissue and the media, was investigated. Liver slices were incubated with Dicumarol for different time periods while oxygen consumption was measured, after which the slices and media were analyzed for Dicumarol. As shown in Table 3, the slices accumulated Dicumarol, so that ultimately there was a greater concentration in the slices than in the media (23). For example, after 75 min, when respiration was depressed about 50%, the slices contained a total of 0.027 mg of Dicumarol, while the medium, which on a volume basis was about ten times as great, contained 0.02 mg. The tissue suspension was estimated to contain about 0.3 g of tissue, and therefore, the amount present in the tissue was estimated to be about 0.090 mg/g, which is about two to five times the amount reported in the liver of

TABLE 3. *Distribution of Dicumarol and Effect on Respiration (23)*

Incubation Time	Amount of Dicumarol			Respiration
	In medium	In slices	Total	
min	*mg*	*mg*	*mg*	*% of control*
3*	0.060	0.010	0.070	100
75	0.022	0.027	0.049	50
180	0.020	0.025	0.045	16

Rat liver slices were incubated with and without 0.075 mg of Dicumarol in a final volume of 3.0 ml for the indicated periods of time. The tissue samples and media were analyzed for Dicumarol by spectrophotometric procedures. * This was the approximate incubation time, representing the time from the initial mixing until the end of the first centrifugation.

treated rats by Green et al. (9), but in the range found in the liver of treated dogs by Weiner et al. (20). It should be pointed out that all of the Dicumarol measured in the slices does not necessarily represent intracellular Dicumarol or uniform distribution within the sample.

The possibility that the cell fractions might bind Dicumarol to different degrees was also considered. In order to determine whether there was preferential binding of Dicumarol by the mitochondrial, microsomal, or soluble fraction, each fraction was dialyzed separately versus Dicumarol, and each of these fractions bound Dicumarol to a marked extent. In view of this, experiments were designed in which Dicumarol was limiting and each cell fraction competed for the available Dicumarol, so that the situation was somewhat more comparable to that within the cell. In these experiments, each of the three cell fractions was dialyzed simultaneously in the same vessel against a limiting amount of C^{14}-labeled Dicumarol. As shown in Table 4, each cell fraction bound Dicumarol, whether total counts or cpm per mg protein are considered (23). If no binding had occurred, the medium would have contained about 150,000 cpm rather than 11,000 cpm. In view of these results, the anticoagulant action of Dicumarol might result either from its interaction with the microsomal fraction, causing a depression of the synthesis of certain proteins, or from its interaction with the mitochondria, thus producing a partial uncoupling with a resultant decrease in ATP levels. In the latter case, systems having the lowest affinity for ATP would be most adversely affected. Finally, the action of Dicumarol might result from its interaction with a soluble enzyme. If these findings can be extended validly to other tissue, then it seems possible that the action of some anticoagulants in producing uricosuric effects (17, 19), or depression of iodide uptake by the thyroid gland (21), might eventually be analyzed in terms of interaction with some of these subcellular components.

The present evidence does not yet permit a conclusion to be drawn as to whether these diverse effects can be explained in terms of a single site of action or in terms of more than one site. In other dialysis studies, purified vitamin K_1 reductase bound Dicumarol, while phosvitin and ovalbumin, for example, did not, which indicates that there is some specificity in the binding. Green et al. (10) had also reported that treatment of animals with Dicumarol did not affect the following systems: DPNH oxidase, aerobic or anaerobic glycolysis, aspartic-glutamic transaminase, ATP-ase, AMP-ase, G-6-P-ase, HDP-ase, xanthine oxidase, pyruvate oxidation, or oxidative phosphorylation.

TABLE 4. *Binding of C^{14}-Dicumarol by Rat Liver Cell Fractions (23)*

Sample	Protein	Radioactivity	
		Total	Specific activity
	mg	*cpm*	*cpm/mg protein*
Dialyzing medium		10,800	
Mitochondria	66	92,150	1400
Microsomes	90	132,700	1490
Soluble fraction	75	104,600	1350

3.0 ml portions of mitochondria, microsomes, and the soluble fraction of rat liver were dialyzed simultaneously against 9.0 ml of 0.25 M sucrose containing 10^{-3} M EDTA, and C^{14}-labeled Dicumarol for 18 hr at 3°C. The dialyzing medium and each cell fraction was then analyzed for radioactivity. The samples containing protein were corrected for the amount of radioactivity not bound by protein.

VITAMIN K_1H_2-CYTOCHROME C REDUCTASE

After the reduction of vitamin K had been studied, it was of interest to determine the fate of reduced vitamin K_1. One possibility considered and investigated was that reduced vitamin K_1 might in turn reduce a cytochrome, such as cytochrome *c*, thus forming reduced cytochrome *c* and regenerating the oxidized form of vitamin K_1. Accordingly, various cell fractions were tested for such catalytic activity. For these studies, reduced vitamin K_1 was formed chemically by reduction with potassium borohydride (KBH_4), rather than by enzymatic procedures. After reduction had occurred, the excess KBH_4 was destroyed with HCl and the samples were kept acidic in order to preserve the reduced form of the vitamin. Such samples, exhibited an ultraviolet spectrum, at neutral pH, similar to the enzymatically reduced vitamin. In addition, after oxidation of the reduced vitamin, it migrated in the same manner as reference samples of vitamin K_1 when tested by paper chromatography. It appeared that there were no major changes in chemical structure resulting from this treatment, but the possibility that changes may have occurred, which could not be detected by spectrophotometric or chromatographic procedures, cannot be excluded. This substrate is, therefore, tentatively designated reduced vitamin K_1.

The standard assay system used for these studies contained citrate-versene buffer of pH 6.2, cyanide to inhibit cytochrome oxidase, oxidized cytochrome *c*, reduced vitamin K_1, and enzyme (25). The reactions were, as a routine, followed by measuring the increase in absorbancy at 550 mμ, which is characteristic of the reduction of cytochrome *c*. There was also a nonenzymatic reduction of cytochrome *c*, which varied with the conditions employed, but corrections were made for this contribution.

In tests of various cell fractions of rat liver, the mitochondrial fraction was found to catalyze the reduction of cytochrome *c*, while the microsomal and soluble fractions contained little or no such activity (Table 1). This activity is tentatively designated vitamin K_1H_2-cytochrome *c* reductase. It may be noted that the cytochrome *c* reductase was localized in the mitochondrial fraction, just as the vitamin K type compounds reported by Green et al. (8), and also by Martius (13). Attempts have been made to solubilize this activity, but to date they have

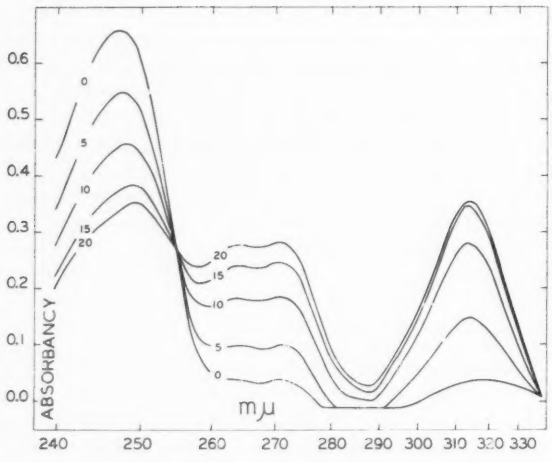

FIG. 3. Spectral changes occurring during the enzymatic reduction of cytochrome *c* by reduced vitamin K_1. The reactions were carried out in citrate-versene buffer of pH 6.2 and recordings were made in a Beckman model DK2 recording spectrophotometer at the indicated time periods in minutes. Reprinted with the permission of Pergamon Press Limited.

met with only partial success. The activity appears to be associated with the mitochondrial membrane or cristae rather than with the matrix, since after freezing and thawing, or after osmotic shock, the activity sedimented at 7,000 × *g*. Treatment of the residue with Lubrol WX caused some solubilization but the activity still sedimented at greater centrifugal forces. Although further treatment with other agents, e.g., digitonin, has been somewhat successful, the yields have been low, and therefore, most of the data presented are concerned with the Lubrol treated samples. Proteins such as bovine serum albumin, crystalline yeast hexokinase, ovalbumin, and phosvitin tested in similar amounts did not catalyze the reduction of cytochrome *c* by reduced vitamin K_1. Whether cytochromes other than cytochrome *c* may serve as electron acceptors in this system has not yet been determined. Before definitive studies are carried out, it would be desirable to obtain fractions which are soluble and free of contaminating enzymes which might interfere.

The effect of the anticoagulants was studied on vitamin K_1H_2-cytochrome *c* reductase. The enzyme was inhibited about 50% by Dicumarol, Sintrom, cumachlor, and phenindione at a concentration of 10^{-4} M, and about 20% by Tromexan and warfarin at the same concentration. In general, vitamin K_1H_2-cytochrome *c* reductase was less sensitive to the anticoagulants than vitamin K_1 reductase.

Antimycin A, which inhibits electron transport between cytochrome *b* and *c*, caused an inhibition of vitamin K_1H_2-cytochrome *c* reductase of about 85% at a concentration of 0.1 μg per ml. Since the reduction of cytochrome *c* was inhibited by Antimycin A, and the details of this reaction are not known, it is possible that a system similar to that described in heart by Ambe and Crane (1), which involves coenzyme Q and is sensitive to Antimycin, may be present in the preparations used and involved in this reaction. Analysis of the preparations obtained to date, has not yet revealed the presence of coenzyme Q, but the possibility that it may be present in minute quantities cannot be excluded. The yields and purity have not permitted a satisfactory analysis for small amounts. It may be mentioned that preparations have been obtained from spinach and dog liver which catalyze this reaction yet do not sediment at 100,000 × *g* (unpublished results). Such preparations may prove to be more suitable for a study of this reaction.

In order to follow the conversion of reduced vitamin K_1 to the oxidized form as cytochrome *c* reduction occurred, some of the reactions were followed in the ultraviolet region, where the spectral changes associated with the oxidation of the reduced form of vitamin K_1 can be followed. However, in order to do this, the system had to be diluted toconditions which were suboptimal so that the ultraviolet spectrum could be measured. The results of such an experiment are shown in Fig. 3. The spectrum of the reduced form is shown by the zero curve, along with the progressive changes occurring after the addition of enzyme. There is less absorption by the reduced form at 275 mμ in Fig. 3 than for the reduced form shown in Fig. 1.

Whether this difference is due to the effect of pH, the nature of the buffer used, or some other factor is being studied. Nevertheless, after the addition of enzyme, there was a progressive decrease in absorption in the 245 mμ region and an increase in the 275 mμ region, both of which are characteristic of the conversion of reduced vitamin K_1 to the oxidized form. In this experiment the nonenzymatic contribution was estimated to be about 30–40% of the overall reaction, which is greater than usual because suboptimal conditions were used so that the spectral changes could be followed. In addition, an increase in absorption was observed in the 315 mμ region, which appears to be due to the reduction of cytochrome *c*, but there are some uncertainties concerning this point. However, there was a correlation between the changes in the ultraviolet and the changes observed at 550 mμ.

DISCUSSION

The natural electron carrier for the two enzymes under investigation requires elucidation in dog and rat liver. However, on the basis of specificity studies, it could be either a substituted naphthoquinone or benzoquinone; or some as yet unidentified substance. On the basis of bio-assay, vitamin K as reported to be present in mammalian tissues and in the case of beef liver, was associated with the mitochondrial fraction (8). But its exact structure was not known. Using C^{14}-labeled menadione, Martius (13), found it to be converted to another substance, which had the highest specific radioactivity in the mitochondrial fraction. Furthermore, in heart, which presumably is not involved in the synthesis of clotting factors, the mitochondria were also found to contain the

labeled material. In subsequent studies (14) a product was isolated and identified as 2-methyl-3-(geranyl-geranyl)-1,4-naphthoquinone, which differs from vitamin K_1 in the side chain at the 3-position. If similar substances are present in dog and rat liver, it is possible that they might serve as electron carriers for the enzymes studied, because in the case of vitamin K_1 reductase there is considerable breadth in specificity. The quinoid portion of the molecule appears to be the functional moiety in oxidation and reduction, because the presence or absence of the side chain at the 3-position does not eliminate activity.

Members of the coenzyme Q series are also associated with the mitochondria and their structure has been determined. But these materials do not possess antihemorrhagic activity in vitamin K deficient animals (5, 12) and, in addition, coenzyme Q_{10} does not act antagonistically to warfarin (12) as vitamin K. On this basis, it would appear that there are at least two types of quinones associated with the mitochondria.

In bacteria, Brodie has reported a soluble factor (2), which participates in electron transport and oxidative phosphorylation with vitamin K being involved (3), presumably in the conversion of inorganic phosphate to an organic form. In the present studies, experiments were carried out to determine whether such a conversion occurred during the reduction of vitamin K_1 or its subsequent oxidation. Up to the present time, no direct evidence has been obtained in the systems under investigation for phosphorylation being associated with these reactions. However, if a free radical or enol type phosphate were involved, the intermediate would probably be unstable. The findings definitely show that substances of the vitamin K type may undergo reduction and, in turn, be oxidized by a cytochrome.

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Role of vitamin K and other quinones in photosynthesis[1]

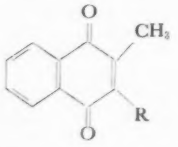
DANIEL I. ARNON

Department of Cell Physiology, University of California, Berkeley, California

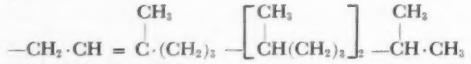
ATTENTION TO A POSSIBLE ROLE of quinones in photosynthesis has been directed by three findings. *a*) Warburg (69) found that isolated chloroplast fragments unable to assimilate CO_2 are still capable of liberating oxygen under the influence of light (Hill reaction), when supplied with substrate amounts of p-benzoquinone (*eq. 1*).

(*1*) $$C_6H_4O_2 + H_2O \rightarrow C_6H_4(OH)_2 + \tfrac{1}{2}O_2$$

b) Dam et al. found (27,27a) that vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone) is localized in chloroplasts.



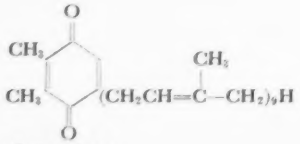
vitamin K_1: R =



menadione (vit. K_3): R = H

phthiocol: R = OH

c) Crane (22), found that a substituted benzoquinone, first isolated from alfalfa by Kofler (42), was characteristically localized in chloroplasts of higher plants. This compound, named by Crane plastoquinone, (also known as Kofler's quinone and Q-254), was found to be a trisubstituted benzoquinone with a 9-isoprenoid side chain. This structure was confirmed by the synthesis of 2,3 dimethyl-5-solanesyl benzoquinone, which was shown to be identical with plastoquinone (43, 60, 66).



plastoquinone

Plastoquinone is closely related structurally to the coenzyme Q (ubiquinone) group, which includes derivatives of 2,3-dimethoxy-5-methylbenzoquinone, substituted at position 6, with a polyisoprenoid side chain (23). Plastoquinone differs particularly from the coenzyme Q homologues in having no methoxy groups.

Naphthoquinones (vitamin K_1) and benzoquinones of the coenzyme Q (ubiquinone) type, of which plastoquinone is an example, have, thus, been found to be normal constitutents of chloroplasts, the cytoplasmic organelles that are now known to be the sites of complete photosynthesis, both inside and, under suitable conditions, outside the living cell (2, 8). Fresh stimulus to investigations of the role of quinones in photosynthesis was given in the last several years by the discovery of photosynthetic phosphorylation by isolated chloroplasts and the involvement of quinone substances in this process. Before discussing the recent developments I shall review briefly some earlier investigations in this field.

EARLY INVESTIGATIONS ON THE ROLE OF VITAMIN K_1 IN PHOTOSYNTHESIS

Interest in a possible association of quinones with photosynthesis was first aroused by the localization of vitamin K activity in green leaves. Almquist (1) observed that the green tops of carrots are a good source of vitamin K, but that the carrot itself contained little or none; Dam (24, 25, 27, 27a) found that the outer, green leaves of cabbage had four times as much vitamin K as the inner leaves. Within the leaf itself, Dam found vitamin K in the chloroplasts, but not in the cytoplasm; in spinach leaves, for example, chloroplasts contained all the vitamin K present in the pressed leaf juice. Dam (26), also demonstrated the presence of vitamin K in *Chlorella vulgaris* and, in smaller concentrations, in four photosynthetic bacteria, including *Rhodospirillum rubrum*.

The form of vitamin K found in green plants, vitamin K_1, partly resembles chlorophyll in containing the phytyl side chain. The synthesis of vitamin K_1 in germinating seedlings parallels that of chlorophyll, as was shown by Dam and his coworkers. Yet, despite this striking association with chlorophyll, the function of vitamin K long remained a mystery. Dam (25), reviewing this subject,

[1] Aided by grants from the National Institutes of Health and the Office of Naval Research.

concluded that the "significance of vitamin K to the function of chloroplasts is just as unknown as the significance of the carotenoids."

Later, in an attempt to test directly the effect of vitamin K substances on photosynthesis, he found that the addition of menadione (2 methyl, 1,4-naphthoquinone) completely inhibited photosynthesis in *Chlorella* whereas Dicumarol, a known vitamin K antagonist, had no effect on either photosynthesis or growth (26). Gaffron (31, 32), who investigated the subject more extensively, found that photosynthesis in another green algae, *Scenedesmus*, was inhibited by low concentrations of menadione and phthiocol. The inhibitory effect of the vitamin K substances appeared, from the results of Gaffron, to center on the oxygen evolution reactions in photosynthesis.

Since Warburg has shown that *p*-benzoquinone (and also β-naphthoquinone sulfonic acid) is a Hill reagent (*reaction 1*), it was natural to suspect that vitamin K_1 in green leaves might be a physiological hydrogen acceptor in that phase of photosynthesis which leads to oxygen evolution. It seemed possible that menadione, which, in the animal, has antihemorrhagic activity equal to that of vitamin K_1, would also be able to function as a Hill reagent with isolated chloroplasts. But the experiments of Arnon and Whatley with menadione as a Hill reagent led to negative results [cf. Arnon (3) p. 502]. Moreover, Wessels (70), in an independent investigation, found that low concentrations of menadione or phthiocol inhibited the Hill reaction.

Wessels (70) explained the inhibitory effects of menadione in his experiments by assuming that it acted in plants in the same manner as dicumarol in animals, i.e., as an "anti-vitamin K" substance that inhibits the natural vitamin K_1 of chloroplasts. This assumption was unsupported by experimental evidence, and ran counter to the well-known effectiveness of menadione as an analogue of the natural vitamin K in the animal. Nevertheless, this interpretation of his results, as well as his comparison of the redox potentials of Hill reagents and vitamin K_1, and other theoretical considerations, led Wessels to suggest that the natural vitamin K_1 in chloroplasts, "is involved in the transfer of hydrogen to Hill oxidants," and that this action is inhibited by menadione.

The first direct experimental evidence that vitamin K substances can act catalytically in stimulating a photosynthetic reaction came after the discovery of photosynthetic phosphorylation by isolated chloroplasts (3, 8, 12). Here, as was later also observed by Wessels (72), menadione and other naphthoquinones acted not as hypothetical antagonists of the natural vitamin K but as powerful catalysts of ATP formation. The catalytic activity of menadione and other naphthoquinones on photosynthetic phosphorylation was inhibited by dicumarol (12), paralleling the antagonistic action of this compound on vitamin K substances in animals. Photosynthetic phosphorylation appeared, therefore, to be the process that was likely to shed light on the role of quinones in photosynthesis. Further research of this question became linked with investigations of the mechanism of photosynthetic phosphorylation.

CYCLIC AND NONCYCLIC PHOTOPHOSPHORYLATION

Photosynthetic phosphorylation (photophosphorylation, for short) is a term coined in 1954 to describe a light-induced ATP formation by isolated chloroplasts, without the aid of mitochondria and without the consumption of oxygen (8). The overall reaction of photosynthetic phosphorylation is represented by *equation 2*:

$$(2) \qquad ADP + P \xrightarrow{\text{light}} ATP$$

Reaction 2, in which the sole product is ATP, was subsequently designated "cyclic photophosphorylation," to distinguish it from a second photophosphorylation reaction by isolated chloroplasts (*eq. 3*), which was found a few years later (13), and named "noncyclic photophosphorylation" (4, 14).

$$(3) \qquad TPN + 2H^+ + ADP + P + 2OH^- \xrightarrow{\text{light}} TPNH_2 + ATP + H_2O + 1/2\ O_2$$

The terms "cyclic" and "noncyclic" refer to the electron flow mechanisms which have been proposed (4, 6) for these two reactions that now jointly constitute the process of photosynthetic phosphorylation. In cyclic photophosphorylation, all of the biochemically effective light energy is used for ATP formation. In noncyclic photophosphorylation, only a portion of the biochemically effective light energy is used for the formation of ATP; the remainder is used for the formation of a reductant, $TPNH_2$ and the excretion (evolution) of oxygen. A non-physiological variant of noncyclic photophosphorylation (*reaction 3*) is *reaction 4*, in which TPN is replaced by ferricyanide (13, 14) (represented here by Fe^{3+}):

$$(4) \qquad 2Fe^{3+} + ADP + P + 2OH^- \rightarrow 2Fe^{2+} + ATP + H_2O + 1/2\ O_2$$

Of the two photophosphorylation reactions (*eq. 2* and *3*), the cyclic type (*eq. 2*), appeared the more basic from the standpoint of mechanisms of energy transformation in photosynthesis when it became clear that it is not limited to green plants but is apparently found in all types of photosynthetic cells. Soon after the discovery of photosynthetic phosphorylation in chloroplasts (8), Frenkel (29), demonstrated photosynthetic phosphorylation in cell-free preparations of the facultatively anaerobic photosynthetic bacterium, *Rhodospirillum rubrum*, and Williams (75), found photosynthetic phosphorylation in the obligately anaerobic photosynthetic sulfur bacteria, *Chromatium* and *Chlorobium*. Thomas and Haans (62), and Petrack and Lipmann (58), found cyclic photophosphorylation in algal preparations (other literature cited in ref. 5).

The unique features of cyclic photophosphorylation are that ATP is formed without the consumption of an exogenous electron donor and an electron acceptor. In respiration, ATP is formed at the expense of free energy released during the flow of electrons from the respiratory substrate to molecular oxygen, both of which are consumed in the process. Cyclic photophosphorylation consumes neither respiratory substrate nor molecular oxygen. The free energy required for the synthesis of pyrophosphate bonds of ATP is supplied solely by light.

Cyclic photophosphorylation in bacteria seemed to differ at first from that in chloroplasts in its dependence on a chemical substrate. Frenkel's cell-free preparations of *R. rubrum* became substrate-dependent after washing; and the rate of phosphorylation was doubled on adding α-ketoglutarate (29). But in subsequent experiments, Frenkel (30) ruled out the dependence on an added chemical substrate and once this fundamental point was clarified, the equivalence of cyclic photophosphorylation in chloroplasts and bacterial particles seemed probable (4).

While cyclic photophosphorylation was recognized as a common denominator of all photosyntheses, noncyclic photophosphorylation as represented by *equation 3*—a reaction that liberates oxygen—seemed at first destined to remain a special feature of plant photosynthesis, since oxygen is not evolved in bacterial photosynthesis. However, Losada et al. (48) (cf. also Duysens et al. (28)) have recently separated reaction 3 into two distinct photochemical reactions; *a*), a photooxidation of hydroxyl ions that yields oxygen (*eq. 5*); and, *b*), a photophosphorylation reaction proper in which the photoreduction of triphosphopyridine nucleotide is coupled with the formation of adenosine triphosphate (*eq. 6*).

(5) $2A^{\ominus} + 2OH^- \xrightarrow{\text{light}} 2A^- + H_2O + 1/2\ O_2$

(6) $TPN + 2H^+ + ADP + P + 2A^- \xrightarrow{\text{light}} TPNH_2 + ATP + 2A^{\ominus}$

(3) Sum: $TPN + 2H^+ + ADP + P + 2OH^- \rightarrow TPNH_2 + ATP + H_2O + 1/2\ O_2$

Only the photooxidation of hydroxyl ions (*eq. 5*) is peculiar to green plants, since only green plants seem capable of using OH^- as an electron donor with the resulting excretion of molecular oxygen. *Reaction 6*, the noncyclic photophosphorylation reaction proper, occurs also in photosynthetic bacteria (57), but they use inorganic or organic electron donors, such as thiosulfate or succinate, instead of OH^-, and hence oxygen evolution does not occur. A comparison of photophosphorylation reactions in green plants and photosynthetic bacteria is given in Table 1.

The discovery of photosynthetic phosphorylation by isolated chloroplasts (8), of both the cyclic and noncyclic type, was confirmed and extended in other laboratories, notably those of Jagandorf (15, 16, 40, 41), Wessels (72, 73), Vennesland (20, 56), and Hill (39). Most of the work has been done with spinach chloroplasts, but recently Whatley et al. (74) have also demonstrated cyclic and noncyclic photophosphorylation in chloroplasts isolated from several other species of plants.

TABLE 1. *Photosynthetic Phosphorylation*

	Green Plant	Photosynthetic Bacteria
noncyclic photophosphorylation:		
a)	$2A + 2OH^- \xrightarrow{Cl^-} 2A^- + H_2O + 1/2O_2$	
b)	$TPN + 2H^+ + ADP + P + 2A^- \rightarrow TPNH_2 + ATP + 2A$	$DPN + 2H^+ + ADP + P + 2A^- \rightarrow DPNH_2 + ATP + 2A$
Sum:	$TPN + 2H^+ + ADP + P + 2OH^- \xrightarrow{Cl^-} TPNH_2 + ATP + H_2O + 1/2O_2$	$DPN + 2H^+ + ADP + P + 2A^- \rightarrow DPNH_2 + ATP + 2A$
cyclic photophosphorylation:	$ADP + P \rightarrow ATP$	$ADP + P \rightarrow ATP$

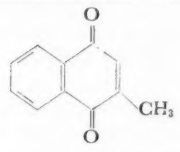
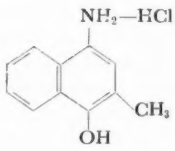
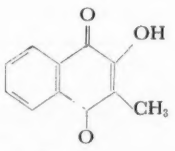
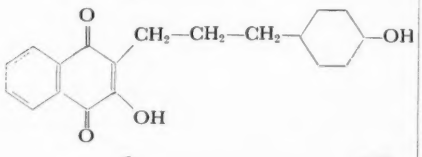
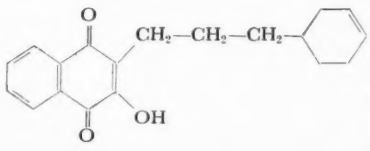
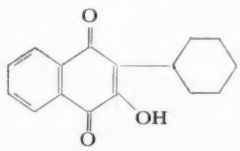
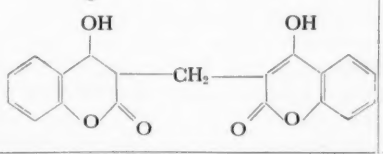
The significance of cyclic and noncyclic photophosphorylation to the overall mechanism of photosynthesis in green plants is that they supply—aside from oxygen as the excreted by-product—the two products, ATP and $TPNH_2$, which alone suffice for converting CO_2 into carbohydrates in the dark (47, 65). Both ATP and $TPNH_2$ (also oxygen), are formed by noncyclic photophosphorylation (*eq. 3*); but cyclic photophosphorylation (*eq. 2*), which supplies only ATP, is also needed for CO_2 assimilation, because the ATP formed in noncyclic photophosphorylation is insufficient to convert CO_2 to carbohydrate (64).

QUINONES AS CATALYSTS OF CYCLIC PHOTOPHOSPHORYLATION

The first indication that quinones may have an effect on photosynthetic phosphorylation came from attempts to circumvent a then puzzling dependence of this process on molecular oxygen. When first discovered, ATP formation by isolated chloroplasts proceeded at a sustained rate only in the presence of oxygen (ref. 8, Fig. 2*b*). However, since there was no net evolution (as measured by manometric pressure change) of molecular oxygen, we concluded then (8), that oxygen acted as a catalyst in photosynthetic phosphorylation and not as a substrate as in oxidative phosphorylation. A decisive difference, then and now, between photosynthetic and oxidative phosphorylation was the inability of chloroplasts to form ATP in the dark by oxidizing substrates of oxidative phosphorylation with molecular oxygen (3, 9).

The dependence of ATP formation by chloroplasts on oxygen was abolished by the addition of catalytic amounts of menadione or other naphthoquinones (12). Photophosphorylation could now proceed at a sustained and vigorous rate in an atmosphere of nitrogen or argon. The process appeared to be anaerobic and, thus, analo-

TABLE 2

Naphthoquinone derivative added	μm P esterified
None	0.8
Vit. K_3 	19.3
Vit. K_5 	19.7
Phthiocol 	19.1
A 	19.3
B 	8.7
C(M-266) 	0.5
Dicumarol 	0.3
Vit. K_3 + Dicumarol	4.8

Effect of vitamin K substances and other naphthoquinone derivatives on photosynthetic phosphorylation. The reaction mixture included a suspension of whole chloroplasts in 0.35 M NaCl containing 0.5 mg chlorophyll, 0.3 μmole of the naphthoquinone derivative added in 0.05 ml methanol; 40 μmole tris (hydroxymethyl) aminomethane buffer, pH 7.2; 20 μmole of a mixture of Na and K phosphate buffer, pH 7.2; 20 μmole of neutralized adenylic acid; 10 μmole sodium ascorbate; and 0.35 M KCl to give a final volume of 3.0 ml. The reaction was carried out in Warburg vessels at 15°; the period of illumination was 1 hr.

gous to photophosphorylations by chromatcphores of photosynthetic bacteria.

The effect of naphthoquinones on photophosphorylation seemed especially interesting at the time because its discovery shortly followed that of Martius on the effect of vitamin K_1 on oxidative phosphorylation by liver mitochondria (50, 51). The effect of naphthoquinones on phosphorylation by chloroplasts was different in that, *a*), chloroplasts were less exacting with regard to the side chain on the third carbon of the naphthoquinone ring. In the experiments of Martius with mitochondria, the phytyl side chain was required; menadione (side chain = H) was inhibitory. Chloroplasts could use equally well menadione, phthiocol (side chain = OH), or other naphthoquinone derivatives (Table 2); *b*), the increase in phosphorylation caused by the addition of catalytic amounts of one of the naphthoquinones was large, of the order of 20-fold (Table 2). Mitochondria, isolated from livers of vitamin K deficient chicks, showed a 30% decrease in oxidative phosphorylation that was restorable in vitro by the addition of vitamin K_1 (51).

Martius (50, 51), has ascribed to vitamin K_1 in oxidative phosphorylation the role of an electron carrier between DPN and cytochromes. We have tentatively proposed an analogous role for naphthoquinones in photosynthetic phosphorylation. In our first working hypothesis, we envisaged naphthoquinones as catalyzing a recombination of the products of photolysis of water (2). We have later abandoned the photolysis of water theory in favor of an electron flow mechanism (4, 6), which agrees better with subsequent experimental findings. The electron flow theory envisages that chlorophyll, on absorbing a photon in the primary photochemical act, expels an electron that has acquired a high energy potential at the expense of the absorbed light energy. Chlorophyll, thus, becomes an electron donor. On losing an electron, chlorophyll becomes also an electron acceptor, i.e., it becomes "electro-positive" [acquires a "hole"; compare the "odd ion" terminology of Lewis and Lipkin (46)].

The "electropositive" chlorophyll compensates for its electron deficiency by accepting an electron from cytochrome which, in turn, becomes oxidized. We have suggested that the electron that has been ejected from the excited chlorophyll passes through a "closed circuit" of the photosynthetic electron transport chain until it finally reaches the oxidized cytochrome. Thus, electrons activated by light and expelled from chlorophyll, return to it by a cyclic path—hence the name cyclic photophosphorylation. During the electron transport steps, coupled enzyme systems convert electron energy into the pyrophosphate bond energy of ATP. A diagram illustrating this concept is shown in Fig. 1.

In the proposed cyclic electron flow mechanism, menadione, or an equivalent quinone, was envisioned as the component of the electron transport chain that catalyzed the transfer of electrons between excited chlorophyll molecules and cytochromes (see "cofactor" in Fig. 1). It seemed that naphthoquinones could also per-

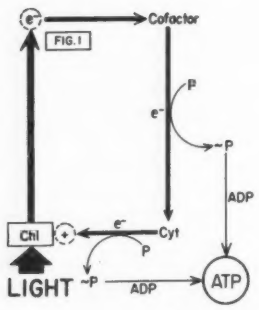


FIG. 1. Scheme for anaerobic cyclic photophosphorylation when the added cofactor is menadione or FMN.

form the same function in photosynthetic bacteria. Geller (33), found that menadione catalyzed photophosphorylation by chromatophores of *R. rubrum*. Moreover, Nozaki, Ogata, and Arnon (reported in ref. 6), obtained evidence with *Chromatium* preparations that naphthoquinones are indeed capable of transferring electrons to cytochromes of photosynthetic bacteria.

In fresh preparations of *Chromatium* chromatophores, the dark reduction of cytochrome c_2, previously oxidized by light, was not influenced by the addition of naphthoquinones. However, as shown in Fig. 2, in aged chromatophore preparations, the reduction of oxidized bacterial cytochromes was greatly accelerated by the addition of naphthoquinones, when added either in the oxidized form (menadione), or in the reduced form (vitamin K_5, i.e., 2-methyl-4-amino-1-naphthol hydrochloride). The effect of naphthoquinones in accelerating the reduction of oxidized cytochrome depended on the presence of chromatophores. Without chromatophores, a 100-fold greater concentration of the reduced naphthoquinone was required to reduce a purified cytochrome c_2 preparation.

Naphthoquinones are not the only catalysts of cyclic photophosphorylation. In isolated chloroplasts, the process is also catalyzed by flavin compounds (FMN, FAD), that are also known to be natural components of chloroplasts. It seemed, at first, that the electron transport in cyclic photophosphorylation involved a joint participation of naphthoquinones and flavin compounds (2), but later investigations in several laboratories (see review, 5), showed that naphthoquinones and flavin compounds can catalyze cyclic photophosphorylation independently of each other. [Baltscheffsky (17), has recently assigned, on the basis of inhibition experiments with atebrin, a key role to flavins as obligatory electron carriers in cyclic photophosphorylation. However, M. Losada, A. Paneque, and A. Mitsui have found in this laboratory, that atebrin, although it effectively uncouples photophosphorylation in chloroplasts, does not inhibit the photochemically induced electron flow.]

The parallel catalytic effects of naphthoquinones and flavin compounds have given rise to questions whether these compounds are to be considered physiological catalysts of cyclic photophosphorylation, or whether they are to be regarded as nonspecific agents in no way distinguishable from nonphysiological catalysts. These questions seemed relevant, because a marked stimulation of photophosphorylation was also obtained by the addition of nonphysiological agents, especially phenazine methosulfate (41). In fact, phenazine methosulfate has been reported to be a better catalyst of cyclic photophosphorylation in chloroplasts than either menadione or FMN (cf. 40).

The superiority of phenazine methosulfate could be explained by its acting as an electron carrier that bypasses a rate-limiting step in photosynthetic phosphorylation (33). On this hypothesis, phenazine methosulfate would give higher rates of phosphorylation than menadione at high light intensity, when there is a rapid flux of electrons from excited chlorophyll. Assuming, however, several sites of phosphorylation in the cyclic pathway, the advantage of phenazine methosulfate might disappear at low light intensity, when the overall rate of the process is limited by the electron flux. Under such conditions, the highest rate of photophosphorylation would be observed in a system in which none of the phosphorylation sites was bypassed. Thus, a comparison of photosynthetic phosphorylation, catalyzed by menadione and phenazine methosulfate under conditions of limiting light, seemed desirable.

The results of such a comparison are shown in Fig. 3. At low light intensity, photophosphorylation catalyzed by menadione was markedly greater than that catalyzed by phenazine methosulfate (or pyocyanin). This difference was persistent and gave a straight-line relationship for a considerable period of time.

These experiments were extended by comparing the rates of photosynthetic phosphorylation at different light intensities. The results shown in Fig. 4 confirm and extend those illustrated in Fig. 3. At low intensity cyclic photophosphorylation catalyzed by either menadione or FMN proceeded at a much higher rate than that catalyzed by phenazine methosulfate. However, at higher light intensities the phenazine methosulfate system gave much greater rates of phosphorylation (cf. 41).

These results suggest that at high light intensity the naphthoquinone (and FMN) systems became limited by enzymatic reactions, which were unable to keep pace with the rapid electron flux. The increasing rates of phosphorylation obtained at high light intensity with the phenazine methosulfate system are consistent with the explanation that this agent does indeed serve as a bypass around some rate-limiting step (6). These findings, although they do not dispose of the question of specificity of naphthoquinones (or flavin compounds), are interpreted as an indication of enzymatic steps that may limit cyclic photophosphorylation at high light intensities, when naphthoquinone (or flavin) catalysts are involved.

The higher rates of photophosphorylation at limiting light with menadione (or FMN) than with phenazine methosulfate, suggest the involvement of at least two phosphorylation sites in the vitamin K (or FMN) p[illegible]

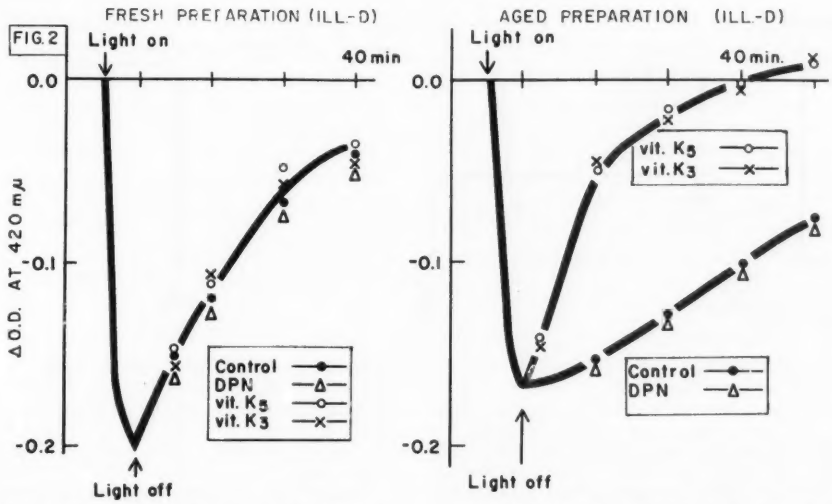


FIG. 2. Effect of vitamin K and other cofactors on the reduction of cytochromes c_2 in cell-free preparations of *Chromatium*. The cytochromes were oxidized by previous illumination. The reaction mixture included in a final volume of 3.0 ml, dialyzed cell-free suspension (PS) containing 0.06 mg bacteriochlorophyll, 0.02 μmoles of purified cytochrome c_2 and 0.03 μmoles of the respective cofactors. Difference in optical density was measured on a Beckman DU spectrophotometer with an attached photomultiplier using cuvettes with the respective cofactor omitted as controls in each case.

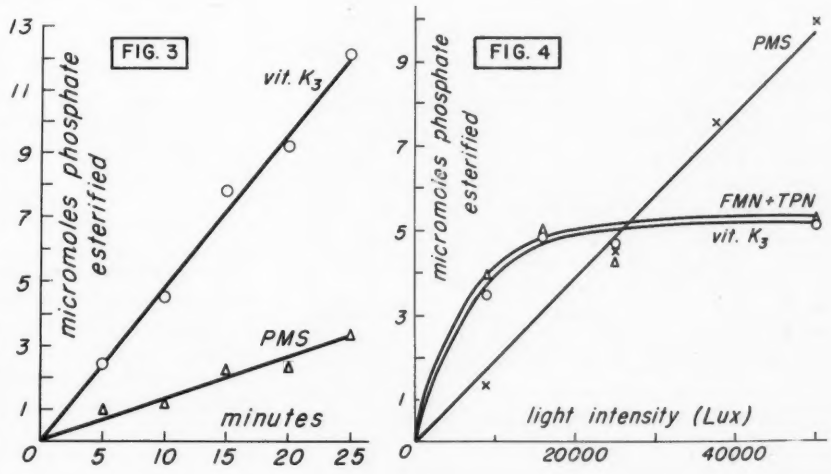


FIG. 3. Effect of menadione (vitamin K_3) and phenazine methosulfate (PMS) on anaerobic cyclic photophosphorylation in spinach chloroplasts at a limiting light intensity (3,000 Lux). Reaction mixture included chloroplast fragments (C_{1s}) containing 1 mg chlorophyll and in μmoles: Tris buffer, pH 8.3, 80; $K_2H^{32}PO_4$, 15; $MgSO_4$, 5; ADP, 15; vitamin K_3 or PMS, 0.3; gas phase nitrogen.

FIG. 4. Effect of light intensity on anaerobic cyclic photophosphorylation. Gas phase nitrogen. Illumination period 30 min. The reaction mixture included chloroplast fragments (C_{1s}) containing 0.1 mg chlorophyll and chloroplast extract equivalent to 1 mg chlorophyll. 0.3 μmoles TPN and 0.3 μmoles FMN were included in the FMN system. Other conditions as given for Fig. 3.

way (Fig. 1). With phenazine methosulfate, there is probably only one phosphorylation site, between cytochrome and chlorophyll (Fig. 5).

A primary phosphorylation reaction, common to all pathways of cyclic photophosphorylation, is envisaged as being coupled with the oxidation of the terminal cytochrome in the photosynthetic particle, i.e., the cytochrome that reacts with the excited chlorophyll molecule (compare Figs. 1 and 5). A second site of phosphorylation in the menadione pathway is likely to occur on oxidation of reduced menadione (or its analogue) by cytochromes, as was suggested by the model reactions proposed by Wessels (70), Harrison (37) and Clark et al. (21).

Although the superiority of menadione (or FMN) over phenazine methosulfate at low light intensity is highly suggestive, it is not considered decisive in answering the question whether vitamin K or flavin compounds, and not other substances normally found in chloroplasts, are the physiological catalysts of cyclic photophosphorylation. The answer to this question must await further experimentation; it cannot be given with assurance on the basis of present evidence.

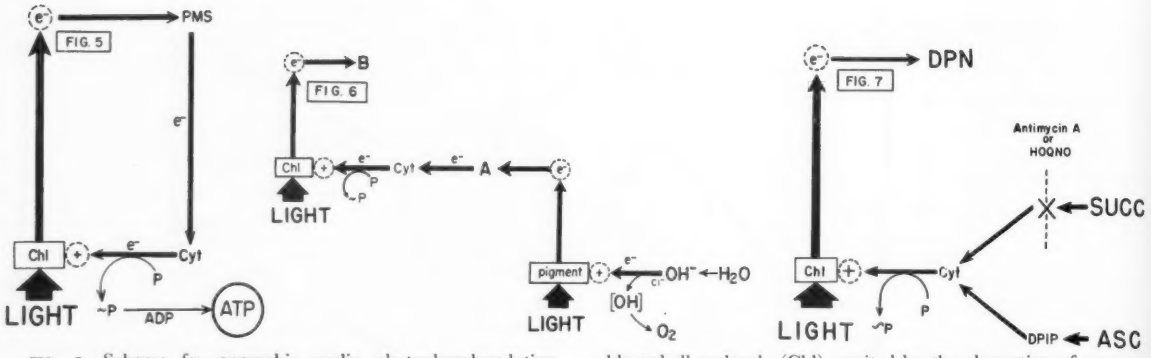


FIG. 5. Scheme for anaerobic cyclic photophosphorylation catalyzed by phenazine methosulfate (PMS).

FIG. 6. Scheme for noncyclic photophosphorylation of the green plant type. In the first light reaction, the pigment molecule becomes excited by the absorption of a quantum of light. The excited pigment donates its high energy electron (e^-) to an intermediate electron acceptor (A) and accepts an electron from an hydroxyl ion. The oxidation product (OH) of the hydroxyl ion is the precursor of molecular oxygen. In the second light reaction, the chlorophyll molecule (Chl), excited by the absorption of a quantum of light, donates its high energy electron (e^-) to the terminal electron acceptor (B) and accepts, via the cytochrome system (Cyt), an electron from the reduced intermediate (A^-) formed in the first light reaction. The phosphorylation step is linked with the transfer of the electron from cytochrome to chlorophyll.

FIG. 7. Scheme for noncyclic photophosphorylation in *R. rubrum*, with either succinate or ascorbate as electron donors.

QUINONES AND NONCYCLIC PHOTOPHOSPHORYLATION

In cyclic photophosphorylation (Fig. 1), menadione acts as an electron carrier and, hence, needs to be added only in catalytic amounts. However, Trebst and Eck (63), in a comprehensive study of quinones in photosynthetic phosphorylation, have recently shown that photophosphorylation of the noncyclic type (*eq. 3* and *4*) occurs when benzoquinones are added to isolated chloroplasts in substrate amounts, i.e., as terminal electron acceptors in a Hill reaction (*eq. 1*). The theoretical stoichiometry of noncyclic photophosphorylation (*eq. 3*), which has also been obtained with ferricyanide (*eq. 4*), is not attainable with benzoquinones, partly because they inhibit the phosphorylation reaction, and partly, because they react back with the liberated oxygen (63).

A special case of photophosphorylation in chloroplasts, involving menadione and other quinones, is the pseudocyclic or oxygen-dependent noncyclic photophosphorylation. Here both menadione and oxygen act as catalysts. The joint catalytic action of menadione and oxygen may be explained by the recently proposed mechanism for noncyclic photophosphorylation in chloroplasts (Fig. 6).

Oxygen, like ferricyanide, can replace TPN, the physiological electron acceptor in noncyclic photophosphorylation (*B* in Fig. 6). Molecular oxygen now acts as a Hill reagent and becomes reduced to hydrogen peroxide [Mehler reaction, (52–54)]. The reduction of oxygen to hydrogen peroxide by illuminated chloroplasts is ordinarily a sluggish reaction, but it is greatly accelerated by menadione or other catalysts (52, 35).

If, at the same time that oxygen is used as the electron acceptor B (top of Fig. 6), OH^- is used as the electron donor (bottom of Fig. 6), the resultant oxygen evolution at one end would balance the oxygen consumption at the other. An oxygen exchange would result ($1/2\ \overset{*}{O}_2$ for $1/2\ O_2$) without any net consumption of oxygen that could be measured manometrically: oxygen would act as a catalyst (*eq. 7*, *8* and *9*).

$$(7)\qquad \overset{*}{O}_2 + 2H^+ + ADP + P + 2OH^- \xrightarrow[\text{menadione}]{\text{light}} H_2\overset{*}{O}_2 + ATP + H_2O + 1/2\ O_2$$

$$(8)\qquad H_2\overset{*}{O}_2 \xrightarrow[\text{endogenous catalase}]{\text{dark}} H_2\overset{*}{O} + 1/2\ \overset{*}{O}_2$$

$$(9)\qquad \text{Sum: } \overset{*}{O}_2 + 2H^+ + ADP + P + 2OH^- \xrightarrow[\text{menadione}]{\text{light}} H_2\overset{*}{O} + 1/2\ \overset{*}{O}_2 + ATP + H_2O\ 1/2\ O_2$$

We have previously called (6) *reaction 9* an "oxygen-catalyzed cyclic photophosphorylation" because its overall balance is the same as that of cyclic photophosphorylation (*eq. 2*). However, *reaction 9* is, in fact, only a special case of noncyclic photophosphorylation, one in which molecular oxygen acts like ferricyanide in replacing TPN as the terminal electron acceptor (*B* in Fig. 6). What marks the pseudocyclic *reaction 9* and points to its having a mechanism different from the truly cyclic *reaction 2* is a dependence on oxygen.

Vennesland et al. (67), have suggested that in chloroplasts menadione usually catalyzes what is called here the oxygen-dependent, pseudocyclic photophosphorylation (*eq. 9*), rather than the truly anaerobic cyclic type (*eq. 2*). Both reactions can be observed in isolated chloroplasts, but under different experimental conditions (10). The two reactions can be readily distinguished experimentally. The pseudocyclic, but not the truly cyclic type, depends on the photoproduction of oxygen (*eq. 7*, *8*, and *9*) and, hence, requires chloride (10, 69), and is sensitive to two well-known inhibitors of oxygen evolution in photosynthesis, CMU (71), (*p*-chlorophenyldimethyl urea), and *o*-phenanthroline (69).

From our point of view, the main interest in menadione as a catalyst of photophosphorylation lies not in its ability to catalyze an electron flow in which oxygen participates as a substitute electron acceptor for TPN (*B* in Fig. 6), but rather in its ability to catalyze a truly anaerobic cyclic electron flow in which oxygen does not participate at all (Fig. 1). The anaerobic cyclic photophosphorylation is a common denominator of plant and bacterial photosynthesis and is, thus, of fundamental importance, whereas the oxygen-dependent pseudocyclic type is merely a special case of noncyclic photophosphorylation in chloroplasts.

EXTRACTION OF QUINONES FROM CHLOROPLASTS

In the investigations discussed so far, the role of quinones in photosynthetic reactions was studied by adding the compound in question to a preparation of isolated chloroplasts. The chloroplasts, themselves, were not treated in any special manner to enhance the effect of the added quinone. Another approach to the study of the role of quinones in photosynthetic reactions has been to extract or treat photosynthetic organelles so as to remove or deplete the native quinone compounds, correlate the treatment with a loss of photosynthetic activity, and restore the lost activity by adding a specific compound.

French and associates (49, 55), observed that the photochemical activity of lyophylized chloroplasts, measured by reduction of 2,6-dichlorophenol during illumination of aqueous suspensions, was largely removed by petroleum ether extraction. The extracted chloroplasts were readily reactivated by β-carotene but, "in every case of reactivation by β-carotene the effect was modest in comparison with the activity of unextracted plastids, as well as with the activation produced by adding back even small amounts of the petroleum ether extract" (55).

Bishop (18), continued these investigations and, by purifying the petroleum ether extract of chloroplasts and commercially available carotene, ruled out the participation of carotenes in restoring the ability of extracted chloroplasts to photoreduce Hill reagents. Instead, he found that "menadione (vitamin K_3) is capable of restoring the photochemical activity (reduction of Hill reagent and oxygen evolution) to extracted chloroplasts under conditions when the effect of purified α- and β-carotene is negligible by comparison." The effect of added menadione or the naphthol derivative, vitamin K_5, was equal to that of the chloroplast extract itself, but the restored activity was still smaller than that of the unextracted control.

In later experiments Bishop (19), found, using a spectrophotometric test, little, if any, vitamin K substances in the petroleum ether extract of chloroplasts. Instead, he found plastoquinone, which Crane (22) has shown to be concentrated in chloroplasts. As for reactivation of the Hill reaction in extracted chloroplasts, Bishop now reported that "qualitatively the effect of the new benzoquinone (plastoquinone) is the same as that of the naphthoquinones (vitamin K, and others). Quantitatively, it is much more effective and, so far, the only compound which produces the same or even better rates of the photochemical reaction than the unextracted controls" (19).

The results of Bishop agree with the findings of Crane that within the leaf plastoquinone is localized in chloroplasts, and support Crane's suggestion that, "plastoquinone is not involved in respiratory electron transport, but that its function should be sought in photosynthetic electron transport" (22). Crane has also recently reported evidence for photoreduction of plastoquinone and photooxidation of the quinol in spinach chloroplasts (23). [There are, nevertheless, some exceptions to the rule that plastoquinone is always associated with chlorophyllous cells. Lester and Crane (45) found small amounts of plastoquinone in corn roots and cauliflower buds. They suggested that this may indicate potential development of chloroplasts in these tissues.] Crane (22) and Bishop (19), found plastoquinone in chloroplasts of leaves and in algae—i.e., in oxygen-evolving cells—but not in photosynthetic bacteria *Rhodospirillum rubrum*, *Rhodopseudomonas spheroides* and *Chromatium*, which carry on photosynthesis without oxygen evolution. Photosynthetic bacteria contain coenzyme Q—an interesting finding, since coenzyme Q is usually associated with cells noted for their aerobic metabolism (45), and photosynthetic bacteria include such strict anaerobes as *Chromatium*.

That the addition of plastoquinone partly restores to chloroplasts, extracted with heptane, the ability to photoreduce a Hill reagent (2,3′,6-trichlorophenol indophenol) has also been recently reported by Krogmann (44). However, Krogmann also found that the heptane-extracted chloroplasts lost most of their capacity for cyclic photophosphorylation of the type catalyzed by phenazine methosulfate. The addition of plastoquinone has, substantially, but not completely, restored this type of photophosphorylation. Krogmann's preliminary publication does not report whether plastoquinone was specific in restoring phosphorylating activity to heptane-extracted chloroplasts.

There are several indications that photophosphorylation in photosynthetic bacteria involves the participation of coenzyme Q and resembles, in this respect, oxidative phosphorylation by mitochondria (36). A stimulation of photophosphorylation by coenzyme Q isoprenologues in chromatophores of *R. rubrum* has recently been reported by Rudney (59). The phosphorylating activity of chromatophores, extracted with petroleum ether, could not be restored by the addition of coenzyme Q, or of the petroleum ether extract itself. However, when the chromatophores were prepared from cells grown in the presence of diphenylamine, the addition of smaller isoprenologues of coenzyme Q (CoQ_2 or CoQ_3) was effective. Such chromatophores had a lower content of coenzyme Q and were evidently structurally altered to be able to respond to added coenzyme Q derivatives.

Support for the participation of coenzyme Q in bacterial photophosphorylations has also come from experi-

ments with inhibitors. Hatefi et al. (38) have observed that antimycin A inhibits the reduction of coenzyme Q by mitochondrial preparations. Antimycin A also strongly inhibits bacterial photophosphorylation in chromatophores of *R. rubrum* (17, 33, 34). In the experiments of Rudney (59), the stimulation of bacterial photophosphorylation by added coenzyme Q_2 or Q_3 was completely inhibited by antimycin A.

Nozaki et al. (7, 57) have recently found with *R. rubrum* chromatophores that, antimycin A [also 2-heptyl-4-hydroxyquinoline-N-oxide (HOQNO); (cf. 17, 61, 68)] inhibits not only cyclic photophosphorylation but also the noncyclic electron transport from succinate, which results in the photoreduction of DPN. This suggests that the cyclic and noncyclic electron transport chain in photosynthetic bacteria have, in common, an antimycin-sensitive site, analogous to that in the electron transport sector of mitochondria, that covers the span between cytochrome *b* and *c*, and where the participation of coenzyme Q is envisaged (36).

In bacterial photophosphorylations, the antimycin-sensitive site can be bypassed both for cyclic photophosphorylation and for noncyclic photophosphorylation. Geller and Lipmann (34), and Baltscheffsky (17), have shown that antimycin inhibition of cyclic photophosphorylation disappears almost completely in the presence of phenazine methosulfate. Likewise, Rudney (59), found that phenazine methosulfate, without the addition of coenzyme Q analogues, has restored photophosphorylation to coenzyme-Q-depleted chromatophores, and that the phosphorylation catalyzed by phenazine methosulfate was relatively insensitive to antimycin A. As for noncyclic photophosphorylation in *R. rubrum* chromatophores, Nozaki, Tagawa, and Arnon (7, 57) have found that, in the presence of antimycin A, ascorbate (together with catalytic amounts of 2-6-dichlorophenol indophenol) served as the electron donor for the phosphorylation that was coupled with the photoreduction of DPN. This noncyclic electron transport bypassed the antimycin-sensitive site (Fig. 7).

We have suggested elsewhere (48), that in chloroplasts a quinone-like substance, perhaps plastoquinone, serves as the link in the electron transport chain (A in *eq. 5* and *6*) between the photooxidation of water (*eq. 5*) and the noncyclic photophosphorylation reaction proper (*eq. 6*). It is possible that in noncyclic photophosphorylation in bacteria the site of coenzyme Q action in the electron transport chain is between the substrate and cytochrome c_2. In cyclic photophosphorylation in bacterial chromatophores, phenazine methosulfate would bypass this site by catalyzing the transfer of electrons from excited chlorophyll to cytochrome c_2 (see Fig. 5).

CONCLUDING REMARKS

The experimental demonstration that isolated chloroplasts are capable of carrying out complete photosynthesis independently of the organization of the living cell (2, 8), has permitted restricting the search for cofactors of plant photosynthesis to the normal constituents of chloroplasts. A consideration of the role of quinones in photosynthesis must begin, therefore, with the question which quinone compounds are localized in chloroplasts?

Until recently this was considered settled by the work of Dam's laboratory, which established that vitamin K is localized in chloroplasts. Dam's determinations of vitamin K were carried out by the curative blood clotting technique with vitamin K-deficient chicks. The validity or specificity of this test for vitamin K substances has not been challenged; plastoquinone has no blood clotting activity.

In the more recent estimations of vitamin K in chloroplasts, which led to negative findings, Crane (22), and Bishop (19), used spectrophotometric methods. Using the same method, Lester and Crane (45), found vitamin K in spinach leaves, alfalfa meal, green, and blue-green algae. Crane (personal communication), suggests that his failure to find vitamin K in chloroplasts may be due to a loss during extraction or because vitamin K in chloroplasts is present in, or converted to, a non-quinoid form, such as naphthotocopherol, which is active in the chick assay but goes undetected by the spectrophotometric test. It seems, therefore, that, assuming equal purity of chloroplast preparations in the experiments of Dam and in those of Crane and Bishop, the negative findings for vitamin K of the latter investigators cannot be regarded as conclusive.

The case for the localization of plastoquinone in photosynthetic tissues in general, and in chloroplasts in particular, has grown stronger since Kofler first described this compound in alfalfa in 1946 (42). The high, though variable, concentration of plastoquinone in leaves—it may approach 0.1–0.2 moles/mole of chlorophyll (23)—would favor an important role for plastoquinone in photosynthesis. Its role seems to be limited however, to plant photosynthesis, in which oxygen evolution occurs. In photosynthetic bacteria Crane (22, 23), found no plastoquinone but coenzyme Q. We have suggested parallel functions for these two substituted benzoquinones in the photosynthetic electron transport chain in green plants and photosynthetic bacteria but final conclusions must await further work. Certain differences may also be noted. Thus, photophosphorylations in bacteria, but not in chloroplasts (9), are sensitive to inhibition by antimycin A.

It is not possible, in the present state of our knowledge, to assess the respective roles of plastoquinone and vitamin K in plant photosynthesis. It is possible that future research will assign specific functions to each in the photosynthetic apparatus of chloroplasts. The intensive investigation of vitamin K compounds in photosynthetic phosphorylation has been facilitated by their availability in a water-soluble form. This was not the case for the coenzyme Q substances. Improved techniques will, no doubt, be evolved which will further advance the study of the role of plastoquinone and coenzyme Q homologues in photosynthetic reactions.

The identification of cyclic and noncyclic photophos-

phorylation in chloroplasts and bacterial chromatophores opened the way for studying the role of catalysts in the photochemical reactions of photosynthesis, without the involvement of the dark reactions of CO_2 assimilation (47, 65). Although it is premature to assign specific roles to individual compounds, present indications are that these photochemical reactions are catalyzed in vivo by one or more of the natural quinone compounds. In the current electron flow theory of the photochemical reactions in photosynthesis (4, 6), quinone compounds assume special interest, because of their ability to accept electrons one at a time, by going through the semiquinone stage. From that consideration alone, either naphthoquinones of the vitamin K type or benzoquinones of the plastoquinone or coenzyme Q types, or both, would be suitable participants in the photosynthetic electron transport chain.

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Personnel mobility of experimental biology

RUTH HABEL

Federation of American Societies for Experimental Biology, Washington D. C.

THE PURPOSE OF THIS REPORT is to present the details of geographic, employer, and primary function changes made by a selected group of 8054 experimental biologists between 1956 and 1958. Detailed personnel data were furnished by individual scientists, in response to questionnaires sent by the Federation of American Societies for Experimental Biology, as one of the participating organizations in the National Register of Scientific and Technical Personnel.

The information received from 8054 individual scientists, all postdoctoral level, in 1956 and again from those same individual scientists in 1958–1959, forms the basis of the study. The degree to which this sample is representative of the national situation has been previously presented.[1] Since 6354 of the 8054 population indicated no pertinent changes over the two-year period, this study is restricted to the 1700 (21%) scientists who have indicated changes in one or more related areas. The majority of geographic changes recorded were from one metropolitan area to another; with a small minority shifting from one institution to another within the same area. Employer category changes involved those scientists who made a definite transfer in type of employer; i.e., University to Private Non-profit Organizations; Federal Government to Industry, etc. The extent to which scientists transferred from one primary function to another was determined by the type of activity reported on the two separate returns in 1956 and 1958 by the individual scientists.

The nature of the changes involved in this study might well be influenced by the stage of the professional career of the individuals involved. This would be reflected by the age distribution of the population which is given below:

Age level	20–29	30–39	40–49	50–59	60+
Percentage of total	2.2	37.5	34.1	15.5	10.7

It is readily seen that the population surveyed is representative of established scientists and includes relatively few in their early postdoctoral years. This should be borne in mind in interpreting the results here presented.

[1] HABEL, R. C. *Federation Proc.* 17: 781–786, 1958.

CHANGES IN STATUS BY FIELDS

Over-all changes. Table 1 summarizes the changes in the three categories of geographic location, employer, and primary function in each of the six major fields of interest of the Federation of American Societies for Experimental Biology. Twenty-one per cent of all scientists in all these fields made some major change in the two-year period. Sixteen per cent changed their geographic location, 9% their employer, and 9% their primary function. The breakdown by fields shows a great deal of mobility in all categories in all six fields but certain ones had more than others.

Under Change in Geographic Location, biochemists and pharmacologists had more changes, while nutritionists and microbiologists had fewer. Change of employer was above average among the biochemists and pharmacologists, but below the average in nutrition and microbiology. A change of function was made more frequently by physiologists, pharmacologists, and pathologists, and less often by biochemists, nutritionists, and microbiologists. The proportion of biochemists, pharmacologists, and pathologists making any kind of change was higher than the average; physiologists were about the average while nutritionists and microbiologists were below the average.

Thus, biochemists, pharmacologists, and pathologists did more shifting than those of other disciplines and nutritionists were the most stable group. Although biochemists changed their geographic location and employer more often, they were more stable as regards a change of primary function.

Change in employer. Table 2 gives the details of changes made by scientists in each of the fields. Under each employer category heading are listed the number of scientists in each field that left that employer category and the number changing to it. The difference between these two figures in reference to the total number of scientists in that employer category in 1956 is the basis for calculating the percentage change. For example, under University, 93 physiologists employed in universities in 1956 changed to a different type of employer,

TABLE 1. *Changes of Status of Experimental Biologists by Fields*

Field	Geographical Change		Employer Category Change		Primary Function Change		Individuals with Any Status Change		All Scientists
	#	%	#	%	#	%	#	%	1956
Physiol.	425	15.6	243	8.9	281	10.3	535	19.7	2712
Biochem.	373	20.5	222	12.2	152	8.3	465	25.5	1818
Pharmacol.	123	19.3	70	11.0	68	10.7	156	24.5	635
Pathol.	119	16.8	68	9.6	83	11.7	163	23.0	706
Nutr.	32	9.6	20	6.0	23	6.9	47	14.2	330
Microbiol.	244	13.1	117	6.3	147	7.9	334	18.0	1853
Totals	1316	16.3	740	9.1	754	9.3	1700	21.1	8054

whereas 89 who worked for an employer other than universities in 1956 shifted to universities. The over-all loss to universities of 4 physiologists is 0.2% of the 1707 physiologists employed in universities in 1956.

In interpreting the results of this detailed breakdown, it must be remembered that in the employer categories State Government and Self Employed, the number of scientists in each field is small so that a transfer of a few individuals is reflected as a relatively large percentage change. For this reason, in these categories individual figures have little or no significance.

It is obvious that the shifts from Universities, and especially from Federal and State Government, to Private Non-profit Organizations, Industry, and Self Employed are generally true in each of the scientific fields. There was more tendency for pharmacologists and microbiologists to transfer away from universities, whereas losses from Federal Government were greatest among the physiologists and pharmacologists. Losses from State Government positions were high in all fields, except physiology and pharmacology. Private Non-profit Institutions gained scientists in all fields, but microbiology was well below the average. Industry increased its employment in all fields, except nutrition and pathology, with major gains in physiology, pharmacology, and microbiology.

In summary, physiologists, biochemists, and pharmacologists tended to leave Government employment and to go into Private Non-profit and Industrial Organizations and Self Employed categories. Microbiologists left Government for Private Non-profit Institutions and Industry.

Changes in primary function. Table 3 shows the details of change of primary function by scientists in each field. The arrangement of this table is similar to that in Table 2 and per cent change is on the basis of number lost or gained in reference to the total number of each t ype o

TABLE 2. *Gain or Loss of Experimental Biologists in Each Field by Employer Category*

Field	University			Federal Govt.			State Govt.			Non-Profit			Industry			Self Employed		
	Transfers		% Change	Transfers		% Change	Transfers		% Change	Transfers		% Change	Transfers		% Change	Transfers		% Change
	From	To		From	To		From	To		From	To		From	To		From	To	
Physiology	93	89	−0.2	80	33	−10.9	7	9	+3.7	40	62	+7.6	9	21	+9.6	14	29	+13.3
Biochemistry	87	85	−0.2	52	37	−5.5	10	2	−25.0	50	72	+8.4	22	23	+0.5	1	3	+9.5
Pharmacology	33	25	−2.3	18	11	−9.2	5	1	−33.3	5	8	+8.3	9	24	+9.7	0	1	+10.0
Pathology	24	23	−0.3	17	9	−6.8	8	3	−13.8	13	25	+9.0	3	0	−7.8	3	8	+38.4
Nutrition	9	7	−0.9	4	3	−2.1	1	0	−20.0	4	9	+27.7	2	0	−4.9	0	1	+12.5
Microbiology	57	36	−2.2	27	17	−2.6	7	5	−1.7	18	22	+2.6	6	35	+11.1	2	2	0
Totals	303	265	−2.1	198	110	−6.7	38	20	−7.2	130	198	+7.7	51	103	+6.1	20	44	+12.4

TABLE 3. *Gain or Loss of Experimental Biologists in Each Field by Primary Function Category*

Field	Research			Teaching			Research Administration			Administration			Clinical Research			Clinical Practice			Consulting			Other		
	Transfers		% Change	Transfers		% Change	Transfers		% Change	Transfers		% Change	Transfers		% Change	Transfers		% Change	Transfers		% Change	Transfers		% Change
	From	To		From	To		From	To		From	To		From	To		From	To		From	To		From	To	
Physiol.	96	110	+1.1	62	66	+0.4	13	47	+22.0	8	17	+10.2	74	1	−55.7	5	35	+17.6	11	5	−21.4	12	0	−48.0
Biochem.	53	67	+1.1	37	43	+2.2	13	29	+10.8	7	9	+3.7	15	1	−50.0	0	2	+22.2	11	1	−55.5	16	0	−37.2
Pharmacol.	25	27	+0.5	19	26	+4.7	3	8	+8.4	1	7	+42.8	13	0	−68.4	1	0	−4.5	0	0	0	6	0	−30.0
Pathol.	10	35	+8.5	12	5	−3.4	5	27	+59.4	5	8	+8.5	35	1	−38.6	4	7	+5.6	8	0	−33.3	5	0	−35.7
Nutr.	8	5	−1.6	5	1	−6.6	3	11	+17.7	3	2	−5.0	2	1	−33.3	0	1	+14.2	1	0	−50.0	0	1	+12.5
Microbiol.	42	55	+1.4	41	28	−2.8	8	39	+19.1	6	18	+8.0	21	0	−37.5	0	6	+17.1	10	0	−28.5	19	2	−28.8
Totals	234	299	+1.5	176	169	−3.6	45	161	+19.2	30	61	+8.6	160	4	−48.0	10	51	+13.8	41	6	−31.2	58	3	−32.5

TABLE 4. *Changes of Status by Employer Category*

Employer in 1956	Geographical Change		Employer Category Change		Primary Function Change		Individuals with Any Status Change		All Scientists
	#	%	#	%	#	%	#	%	1956
University	682	14.9	303	6.6	391	8.5	905	19.8	4568
Fed Govt.	301	22.8	198	15.0	142	10.7	355	26.9	1315
State or Local Govt.	44	17.5	38	15.1	39	15.5	60	23.9	251
Private Nonprofit Organization	170	19.2	130	14.7	91	10.2	203	22.9	884
Industry	97	11.5	51	6.0	71	8.4	151	17.9	843
Self Employed	22	11.3	20	10.3	20	10.3	26	12.0	193
Totals	1316	16.2	740	9.1	754	9.3	1700	21.0	8054

TABLE 5. *Number of Experimental Biologists Transferring from and into Indicated Employer Categories*

Employer	Univ.	Federal Govt.	State Govt.	Private Nonprofit Organ.	Industry	Self Employed	Total Loss	% Gain or Loss	% Turnover
University		81	16	126	62	18	303	−2.1	12.4
Federal Govt.	121		2	51	19	5	198	−6.7	23.4
State Govt.	16	4		11	2	5	38	−7.2	23.1
Private Non-profit Organization	75	21	1		19	14	130	+7.7	37.1
Industry	37	4	1	7		2	51	+6.1	18.2
Self Employed	16	0	0	3	1		20	+12.4	33.1
Total gain	265	110	20	198	103	44	740		

scientists in a given field in 1956. The numbers involved in some groups are too small to be significant in themselves so only trends may be indicated.

All fields, except nutrition, showed a slight increase in those engaged in Research. Biochemistry and pharmacology had an increase in the number of teachers, while pathology, nutrition, and microbiology showed a decrease. Research Administration had substantial increases in all fields as did General Administration with the exception of nutrition. There was a marked shift away from Clinical Research in every field, with a corresponding increase in Clinical Practice in every instance except pharmacology. Although there were small numbers in the Other category, which included such functions as Design and Development and Biologics Control; there was a general tendency for a loss.

In summary, physiologists tended to leave Clinical Research, Consulting, and other functions for Research, Administration, and Clinical Practice. Biochemists were leaving the same areas to go into Research, Teaching, and Administration, more particularly Research Administration. Pharmacologists were transferring from the same functions but were entering Teaching and Administration, more so to General Administration. Pathologists shifted from Teaching, Clinical Research, Consulting, and Other, to Research, Research Administration, and Clinical Practice. Nutritionists were the only group showing a loss for Research which, combined with a loss from Teaching, showed a shift into Research Administration. Microbiologists were leaving Teaching, Clinical Research, Consulting, and Other for Research, Administration, and Clinical Practice.

CHANGES IN STATUS BY EMPLOYER

Over-all changes. Table 4 summarizes the changes in the three categories of geographic location, employer, and primary function in each of the six employer cate-

TABLE 6. *Over-all Gain or Loss of Experimental Biologists in Primary Function by Employer Category*

Employer	Research			Teaching			Research Administration			Administration			Clinical Research			Clinical Practice			Consulting			Other		
	Transfers		% Change	Transfers		% Change	Transfers		% Change	Transfers		% Change	Transfers		% Change	Transfers		% Change	Transfers		% Change	Transfers		% Change
	From	To		From	To		From	To		From	To		From	To		From	To		From	To		From	To	
University	139	138	−0.04	162	156	−0.3	7	49	+36.5	13	24	+7.9	58	2	−61.5	0	11	+17.7	8	1	−38.8	8	0	−33.3
Federal Govt.	41	54	+1.5	6	1	−22.7	16	39	+11.6	7	16	+13.4	42	0	−48.8	0	6	+25.0	13	1	−36.3	18	0	−52.9
State Govt.	4	5	+1.3	0	5	+55.5	3	16	+59.0	2	6	+5.8	19	0	−57.5	0	1	+20.0	6	0	−33.3	3	1	−11.1
Private Nonprofit Organization	26	46	+3.5	4	4	0	5	29	+34.2	3	11	+18.1	33	1	−34.0	2	14	+21.8	8	0	−50.0	8	1	−38.8
Industry	23	52	+5.7	2	0	−100.0	14	28	+7.0	5	4	−2.5	6	0	−37.5	1	2	+10.0	4	1	−27.2	18	1	−32.7
Self Employed	1	4	+30.0	2	3	+33.3	0	0	0	0	0	0	2	1	−20.0	7	17	+7.1	2	3	+6.2	3	0	−17.6
Totals	234	299	+1.5	176	169	−0.3	45	161	+19.2	30	61	+8.6	160	4	−48.0	10	51	+13.8	41	6	−31.2	58	3	−32.5

TABLE 7. *Changes of Status by Primary Function Category*

Primary Function 1956	Geographical Change		Employer Category Change		Primary Function Change		Individuals with Any Status Change		All Scientists
	#	%	#	%	#	%	#	%	1956
Research	131	9.4	66	4.8	43	3.1	172	12.3	1397
Teaching	181	9.4	96	5.0	176	9.3	267	13.9	1919
Res. Admin.	68	11.2	35	5.7	45	6.2	84	13.9	604
Admin.	28	7.7	9	2.5	30	10.3	55	15.3	359
Clin. Res.	104	32.0	82	25.2	160	49.2	192	59.0	325
Clin. Prac.	17	5.7	12	4.0	10	3.3	18	6.0	296
Consulting	22	19.0	13	11.6	41	34.8	46	41.0	112
Other	23	13.6	8	4.7	58	34.3	60	35.5	169
Totals	1316	16.2	740	9.1	754	9.2	1700	21.0	8054

gory in 1956 represents the percentage gain or loss. Column 10 (% turnover) is the sum of those leaving and entering a particular type of employment in relation to the total in 1956.

Of the 303 scientists leaving Universities for other employment, most went to Private Non-profit Institutions with significant numbers entering Government and Industrial Organizations and to Self Employed. At the same time there was a great deal of exchange between different employer categories; i.e., between Private Non-profit, Universities, and Federal Government. Throughout this report, by Employer Change is meant the change in employer category. Certainly in the case of those employed in Universities there were a great number of changes of employment to a different university, but these changes were not included in this analysis.

Research, Teaching, Res. Admin., Admin., Clin. Res., Clin. Prac., Consulting, Other, Totals

Research 131 66 4 0 31 2 0 234 +1.5 12.4
Teaching 106 38 19 2 8 1 2 176 −0.3 17.9
Res. Admin. 29 7 4 0 2 2 1 45 +19.2 34.1
Admin. 20 2 5 1 2 0 0 30 +8.6 25.3
Clin. Res. 92 21 31 16 0 0 0 160 −48.0 50.4
Clin. Prac. 2 3 0 5 0 0 0 10 +13.8 20.6
Consulting 20 1 8 7 1 4 0 41 −31.2 41.9
Other 30 4 13 6 0 4 1 58 −32.5 36.0
Total gain 299 169 161 61 4 51 6 3 754

In summary, Universities and Government lost post-doctoral level biological scientists to Private Non-profit Organizations. Those leaving Private Non-profit went chiefly to Universities but also to Federal Government, Industry, and Self Employed. Industry's major loss was to Universities, as was also those originally Self Employed.

TABLE 8. *Gain or Loss of Experimental Biologists in Each Employer Category by Primary Function*

Primary Function	University			Federal Govt.			State Govt.			Non-Profit			Industry			Self Employed		
	Transfers		% Change	Transfers		% Change	Transfers		% Change	Transfers		% Change	Transfers		% Change	Transfers		% Change
	From	To		From	To		From	To		From	To		From	To		From	To	
Research	199	196	−0.1	138	88	−5.8	20	9	−14.1	93	138	+8.0	38	90	+10.2	3	2	−10.0
Teaching	73	46	−1.4	5	1	−18.1	1	4	+33.3	3	12	+31.0	0	0	0	3	3	0
Res. Admin.	6	9	+2.6	13	14	+0.5	3	3	0	6	20	+20.0	7	10	+1.5	0	1	+100.0
Administration	2	2	0	5	5	0	4	3	−1.4	1	7	+13.6	0	1	+2.5	0	0	0
Clin. Res.	15	2	−14.2	24	0	−27.9	8	0	−24.2	16	4	−12.7	2	0	−12.5	8	2	−75.0
Clin. Practice	6	10	+6.4	5	2	−12.5	1	1	0	6	15	+16.3	0	0	0	3	27	+17.8
Consulting	1	0	−5.5	3	0	−9.0	1	0	−5.5	4	1	−18.7	3	0	−27.2	1	8	+43.7
Other	1	0	−4.1	5	0	−14.7	0	0	0	1	1	0	1	2	+1.7	2	1	−5.8
Totals	303	265	−2.1	198	110	−6.7	38	20	−7.2	130	198	+7.7	51	103	+6.1	20	44	+12.4

gories. The change of geographic location was greatest in those employed by the Federal Government, with State Government and Private Non-profit Organization employees also above average. Industry and Self-employed categories were well below average. Those working for Federal and State Government and Private Non-profit Organizations had the highest rates of change of employer, with University and Industry well below average. A change of primary function took place more often in Government and Private Non-profit Organization employees, although Universities and Industry were not very much below average. The proportion of individuals making any kind of change was high in all employer categories, being highest in Federal Government and well below average in Self Employed.

Changes in employer. Table 5 gives the details of the employer changes made by those in each employer group. This table is arranged so that column 8 (Total Loss) shows the total number of scientists leaving the indicated employer and the horizontal lines indicate the new employer to which these people transferred. The horizontal line at the bottom of the table, therefore, gives the total number transferring to the indicated employer. The difference between total loss and total gain in reference to the number in each employer cate-

Changes in primary function. Table 6 gives the details of changes of primary function by experimental biologists in each employer category. The arrangement of this table is similar to Table 3.

All employer categories, except Universities, showed a slight increase in those engaged in Research (1.5%). Under Teaching, obviously those employed by Universities represented the only group with significant numbers and there was practically no change. Research Administration showed substantial gains in every major employment type, as did General Administration in all but Industry, with over-all increases of 19% and 8% respectively. Clinical Research, Consulting, and Other functions lost personnel in every employer situation while Clinical Practice gained in all. The over-all loss from Clinical Research represented 48%.

In summary, University biologists tended to shift from Clinical Research into Research Administration, General Administration, and Clinical Practice. The same trend existed in all five major categories with minor exceptions. Of particular interest is the tendency for marked increases in Research Administration, General Administration, and Clinical Practice, with those in Universities, State Government, and Private Non-profit Organizations being well above average in this shift.

CHANGES OF STATUS BY PRIMARY FUNCTION CATEGORY

Over-all changes. Table 7 is similar in arrangement to Tables 1 and 4. An unusually high degree of geographic mobility occurred in Research, Clinical Research, and Consulting groups. These same groups also changed employers at a high rate, while those in the remaining functions were well below the average in this type of shift. Primary function changes were quite common among those engaged in Clinical Research, Consulting, and Other; with Teaching and General Administration, average; and Research, Research Administration, and Clinical Practice below average.

In summary, Clinical Research and Consulting recorded an extremely high degree of mobility in all categories. Teaching, Research Administration, General Administration, and Clinical Practice were average or below average in all instances. The Research group, on the other hand, showed higher than average mobility from the standpoint of Geographic and Employer Change but were less likely to change their Primary Function.

Changes in employer. Table 8 shows employer changes that occurred in the different primary function groups. The University population showed a slight decrease in all areas, with the exception of Research Administration and Clinical Practice. There was a tendency for experimental biologists in all functions, except the two types of Administration, to transfer from Federal Government. Private Non-profit Institutions and Industry gained personnel in most functions, the losses being in Clinical Research and Consulting. The Private Non-profit Organizations gains were least in Research, whereas this function showed the largest gain in Industry.

In summary, the primary function that had a consistent gain in all employer situations was Research Administration, whereas Clinical Research lost personnel markedly in all Employer Categories. Personnel in Research tended to leave Government for Private Non-profit Organizations and Industry.

Primary function changes. Table 9 is similar in arrangement to Table 5, in that Column 10 (Total Loss) shows the total number of scientists leaving the indicated function and the corresponding horizontal line indicates the functions to which they transferred. The horizontal line at the bottom of the table, therefore, gives the total number transferring to indicated functions.

Of the 234 scientists transferring away from Research, the majority entered Teaching and Research Administration. The greater proportion of those leaving the teaching profession transferred to Research and Research Administration. A small number of scientists left Research Administration for Research, as did those in General Administration. Clinical Research lost 48% of its personnel, most of whom went into Research or Research Administration. Clinical Practice shows a gain; but here, again, numbers involved are so small as to be of doubtful value. Consulting and Other showed a significant loss, mostly to Research.

In summary, Research Administration evidenced a substantial gain, and Clinical Research a considerable loss, as did Consulting and Other. The Research and Teaching personnel were fairly stable.

SUMMARY

The six major fields of experimental biology at post-doctoral level represented in this study show a high degree of mobility between different geographic areas, type of employer, and primary function. Of the same 8054 respondents in 1956 and 1958, 21% indicated one or more changes.

Changes in geographic location and employer category were made more frequently by the biochemists and pharmacologists. Physiologists indicated the highest rate of function change, while nutritionists and microbiologists experienced the least change in all areas.

Private Non-profit and Industrial Organizations showed the greatest gains in scientific personnel; Federal Government, the greatest loss.

Research Administration recorded substantial gains, while Clinical Research indicated a sharp decrease. Research and Teaching remained fairly stable.

A definite shift into Research Administration and Clinical Practice was apparent, as was also a shift from Clinical Research. The groups engaged in Research and Teaching remained fairly constant in size.

Society member interchange at Federation scientific sessions

FEDERATION STAFF STUDY

UNDER THE DIRECTION of the Federation and with the cooperation of all societies, a study was made of the attendance at the 1961 Federation Meeting. In addition to a detailed check of registration cards, each society was assigned a color for the badges of its members and non-members with a major interest in that particular society. The colored badges permitted a quick count of various society affiliates attending any particular sessions, with the tabulation reported by the chairman. A third check on attendance was made by requesting registrants to return postcards indicating the numbers of the sessions they attended.

The analysis of the tabulations gave a good indication of points that need to be studied in greater detail. This study should be part of an investigation of the problem of oral communication between scientists. Some of the questions that should be answered will be raised after a brief presentation of the tabular data that are now available.

REGISTRATION

There was a total registration of 12,577 at the 1961 Federation Meeting. Of these, 2,852 were members; 6,354 non-member scientists; and 1,420 graduate students. There were also 732 guests, 68 representatives of the press, and 1,151 exhibitors. Tables 1 and 2 show the breakdown of the registration of scientists at the meeting. Only 27% of the registered scientists were members of the Federation. The percentage of members of each society attending the meeting is calculated from the attendance, plus those with dual membership.

ANALYSIS OF POSTCARD DATA RESPONSE

Table 3 gives a breakdown of the number of cards received, the number that could be used, and the percentage of usable returns from those registered. Some cards were not usable because the session numbers were not marked properly—society numbers only. Several that showed paper numbers, rather than session numbers, were decoded and used.

In order to have comparable ratios between societies for judging crossover a weighting factor was used. This factor was multiplied by the actual number of attendances (taken from the cards) to determine the weighted attendance at any session or groups of sessions. This weighted attendance was used for all the calculations of attendance ratios.

It will be noted that the return from members (66%) was quite good, especially for the two largest societies (25%), Physiology and Biochemistry. The return from non-members was expectedly much lower; in fact, too low to permit any significant calculations or conclusions.

INDIVIDUAL CROSSOVER

To obtain a general idea of what people as individuals did, the cards were sorted into those attending only sessions of their society affiliation, plus Intersociety Sessions, and those that showed a mixed attendance at various society sessions.

Tables 4 and 5 show the percentage of crossover for both members and non-members.

It will be noted that the two largest societies showed less crossover than the other societies, both for members and non-members. Even with the largest societies the crossover by individuals was quite significant at approximately 50%.

ATTENDANCE CROSSOVER BY SOCIETIES

All data in the remaining tables were taken from member cards only, since it was felt that the percentage return of non-member cards was not high enough to warrant analysis.

In order to determine where members went, the weighted attendance figures for members of each society were compared for society crossover and also for area of interest.

Table 6 shows the ratio of society member attendance at the various society sessions. As expected, Intersociety sessions showed a fairly good attendance from all groups.

Each society supported its own sessions to a fairly high degree. This was especially true of Physiology, Biochemistry, and Immunology.

Physiologists were interested in programs in Biochemistry and Pharmacology; Biochemists in programs in Physiology and Nutrition; Pharmacologists in programs in Physiology and Biochemistry; Pathologists in Immunology, Biochemistry, and Physiology; Nutritionists in programs in Biochemistry and Physiology; and Immunologists in Biochemistry and Pathology.

MAKEUP OF SESSION ATTENDANCE

Table 7 shows the ratios of attendances at various sessions; i.e. the per cent at any session by societies.

The largest percentage of attendees at any session was that of the sponsoring society. There were members of every society at sessions of all societies, except that there were no Nutritionists at Immunology sessions.

TABLE 1. *Member Registration*

	Members	Dual Membership	Dual Membership Prorated	% Prorated Society Membership Registration	% of Total Membership Registration	% of Total Scientific Registration
More than one Society, paid registrations	372					
Physiology	771	(157)	848	42	30	8
Biochemistry	784	(201)	883	55	31	8
Pharmacology	342	(149)	416	46	15	4
Pathology	191	(43)	212	33	7	2
Nutrition	201	(161)	280	38	10	3
Immunology	191	(45)	213	35	7	2
Total for Fed.	2852	(756)	2852		100	27

TABLE 2. *Non-Member Registration*

	Non-members	Non-members Dual Interest	Graduate Students	Graduate Students Dual Interest	Total Non-Members	% of Total Scientific Registration
More than one Society, paid registrations	694		111		805	8
Physiology	1,381	(230)	346	(55)	1,727	16
Biochemistry	2,535	(441)	560	(69)	3,095	29
Pharmacology	679	(183)	206	(20)	885	8
Pathology	312	(104)	73	(16)	385	4
Nutrition	177	(141)	42	(34)	219	2
Immunology	576	(130)	82	(19)	658	6
Total for Fed.	6,354	(1,229)	1,420	(213)	7,774	73

The table shows some indication of paired interests—Physiology and Pharmacology; Biochemistry and Nutrition; Pathology and Immunology. The geographical location of the sessions may have influenced the extent of crossover in some cases.

CROSSOVER BY AREA OF INTEREST

Since the data showed a fair amount of crossover it was of interest to determine if the crossover was due primarily to an area of common interest. The 270 sessions were combined into groups showing relatively common interest regardless of society sponsorship.

Table 8 shows the ratio of attendees at various sessions, i.e. the percentage of attendance at a particular category of sessions by societies.

Again this pairing of interest shows up in 21 out of 33 cases. And again it proved that the largest attendance is by members of the major sponsoring society or societies. There is, however, a great deal of crossover and some indication that an integrated Federation program might be helpful.

The data give some guide lines for planning future Federation meetings, whether they be integrated, staggered by societies over a longer period of time, or divided into groups of societies or groups of area interests.

Table 9 shows the ratio of area interest of members attending the meetings. Only 2% or more of member attendance is recorded. This table shows the major areas of interest of various members. It shows the ratio of attendances of any particular society's membership at the various sessions of a specific interest. It also indicates what their major interests are.

BADGE COUNT

A total of 20 chairmen did not submit reports of their sessions and 23 of the 250 reports received did not contain sufficient data on attendance. Many of the sessions were too large to make a count of badges practical, and in several rooms, due to pillars and other obstacles,

TABLE 3. *Response*

Society	Members					Non-Members				
	Members registered*	Member cards received	Usable member cards	Weighting factor to compare session attendance	% of registered members who replied	Non-members registered	Non-member cards received	Usable non-member cards	Weighting factor to compare session attendance	% of registered non-members who replied
Physiol.	848	689	654	1.00	77	1727	509	485	1.76	28
Biochem.	883	671	629	1.04	71	3095	910	854	1.00	28
Pharmacol.	416	250	225	2.91	54	885	216	202	4.23	23
Pathol.	212	126	116	5.64	55	385	96	74	11.54	19
Nutr.	280	152	135	4.84	48	219	76	66	12.94	30
Immunol.	213	152	130	5.03	61	658	179	148	5.77	22
Fed. at large						805	147	135	6.33	17
Totals	2852		1889		66	7774		1964		25

* Dual memberships prorated.

TABLE 4. *Member Crossover**

Society	Attended only Soc. and Intersoc.	% That did not mix	Mixed attendance (crossover)	% That mixed	Totals
Physiol.	313	48	341	52	654
Biochem.	346	55	283	45	629
Pharmacol.	60	27	165	73	225
Pathology	22	19	94	81	116
Nutrition	32	24	103	76	135
Immunology	56	43	74	57	130
Totals	829	44%	1060	56%	1889

* Determined by checking sessions on cards.

TABLE 4a. *Extent of Individual Member Crossover*

Society	Number of Other Society Sessions Attending						Total Number (Table 4)
	1	2	3	4	5	5 or more	
Physiol.	33%	24	12	10	7	14	341
Biochem.	47	19	14	7	3	10	283
Pharmacol.	27	16	17	13	8	19	165
Pathology	16	20	16	18	14	16	94
Nutrition	27	18	16	15	8	16	103
Immunology	14	26	12	4	5	16	74

Example: 47% of the individual members of Biochemistry who mixed, attended only one other session than Biochemistry or Intersociety.

Physiology, Biochemistry and Immunology showed the least amount of crossover (Table 4). A large percentage of those who crossed over, did so for only one session (Table 4a).

TABLE 5. *Non-Member Crossover**

Society of major interest	Attended only Soc. and Intersoc.	% That did not mix	Mixed attendance (crossover)	% That mixed	Totals
Physiol.	260	54	225	46	485
Biochem.	419	49	435	51	854
Pharmacol.	74	37	128	63	202
Pathology	13	18	61	82	74
Nutrition	19	29	47	71	66
Immunology	64	43	84	57	148
Fed. at Large			135	100	135
Totals	849	43%	1115	57%	1964

* Determined by checking sessions on cards.

accurate counts were impractical. There were, however, several very accurate counts made by chairmen, but there could be no differentiation between members and non-members.

The reports did give a very clear indication of crossover, and the colored badges served the purpose at the meeting of making people aware of the amount of crossover, particularly in certain sessions.

TABLE 6. *Attendance Crossover by Societies (Where did Society Members go?)**

Society Attendees	Sessions						
	Intersoc	Physiol.	Biochem.	Pharmacol.	Pathol.	Nutr.	Immunol.
Physiol.	19%	61	10	4	3	1	1
Biochem.	14	5	71	3	1	4	2
Pharmacol.	31	16	10	38	3	1	1
Pathology	23	8	10	3	39	2	14
Nutrition	20	6	20	1	2	50	0
Immunology	12	1	10	2	4	1	70

* Read across. Example: 16% of total weighted Pharmacology attendees were at Physiology meetings.

TABLE 7. *Makeup of Session Attendance (% of Session Attendance by Societies)*

Society Attendees	Sessions						
	Intersoc.	Physiol.	Biochem.	Pharmacol.	Pathol.	Nutr.	Immunol.
Physiol.	16%	62	7	8	5	1	1
Biochem.	12	5	56	5	3	7	2
Pharmacol.	26	17	7	75	6	1	2
Pathology	18	8	7	5	73	3	15
Nutrition	17	6	15	3	4	87	0
Immunology	10	1	7	3	8	0	79

* Read down. Example: 15% of the weighted attendance at Biochemistry sessions were Nutritionists.

Examples of crossover, as reflected in the midway session count from the chairmen's reports, show that in some cases the attendance from other disciplines was substantial.

COMMENTS FROM CARDS

Comments written on the returned cards were grouped into four categories: *1*) remarks relative to the size of the meeting; *2*) those relative to an integrated Federation program; *3*) comments on sessions; and *4*) general observations. The following summarizes the major comments.

1) Most comments indicated dissatisfaction with the size of the meeting and the number of papers. Suggestions included some to reduce the number of 10-min papers by editing or permitting a member to sponsor a paper only every other year; reducing the number of 10-min papers and having more symposia and panel discussions.

2) Many members felt that the Federation program should be arranged by topics and not by societies. This might avoid some of the conflicts of interest in sessions. There was also the feeling that it was advantageous to have all societies meet together if this was at all possible.

3) Many felt that the exhibits and movie sessions should remain open evenings, with a printed time

TABLE 8. *Area of Interest (% of Session Attendance by Societies)*

General Area of Interest	No. of Individual Sessions	Society					
		Physiol.	Biochem.	Pharmacol.	Pathol.	Nutr.	Immunol.
Amino Acids	7	2	*29*	2	3	*62*	2
Antibodies	14	1	3	2	*18*	1	*76*
Blood	11	14	11	6	*43*	6	*20*
Carbohydrates	8	10	*55*	7	7	*18*	2
Cardiovascular	24	*28*	6	*27*	21	17	1
Cellular & Tissue	13	5	11	12	*36*	4	*32*
Drugs	4	7	13	70	4	6	0
Electrolyte & Trans.	6	37	30	16	3	12	2
Endocrines	15	*34*	20	*21*	15	8	2
Environ. Physiol.	6	63	3	12	2	20	0
Enzymes	18	6	*57*	8	1	*25*	3
Gastrointestinal	12	15	8	11	42	23	1
Genetics	2	3	53	9	9	5	21
Lipids & Steroids	13	7	*32*	6	8	*46*	1
Mineral & Bone	3	4	4	2	2	88	0
Muscle	5	*57*	12	*23*	4	4	0
Neuropharmacology	20	*9*	5	*82*	1	2	1
Neurophysiology	10	*66*	3	*23*	4	1	3
Nucleic Acids	8	6	58	8	8	8	11
Proteins	12	5	*50*	3	9	*20*	13
Radiation & Radiobiology	3	22	17	12	32	13	4
Renal & Water	10	34	3	23	34	5	1
Respiration	9	*83*	1	*8*	3	3	1
Toxicology	4	9	7	*58*	18	3	5
Tumors & Cancer	6	3	6	6	*50*	1	*34*
Viruses	7	1	3	2	*20*	1	*73*
Vitamins	4	1	*10*	1	1	*87*	0
Physiol. General	5	*51*	8	*15*	9	15	2
Biochem. General	4	10	*49*	14	6	*15*	6
Nutrition General	4	1	7	1	3	*87*	1
Pharmacol. General	1	0	7	55	9	0	29
Pathol. Movies	1	7	7	0	85	0	0
Fed. Movies	1	*30*	8	*28*	13	21	0

Read across. Example: 18% of those attending the antibody sessions were Pathologists.

TABLE 9. *Ratio of Area Interest of Members (2% or more)*

Area of Interest	No. of Sessions	Members					
		Physiol.	Biochem.	Pharmacol.	Pathol.	Nutr.	Immunol.
Amino Acids	7		4			9	
Antibodies	14		2		11		44
Blood	11	2	2		7		3
Carbohydrates	8		6			2	
Cardiovascular	24	17	3	16	13	10	
Cellular & Tissue	13	2	3	3	11		9
Drugs	4			4			
Electrolyte & Trans.	6	5	4	2		2	
Endocrines	15	11	6	6	5	2	
Environ. Physiol.	6	4					
Enzymes	18	2	14	2		7	
Gastrointestinal	12	4	2	3	12	6	
Genetics	2		2				
Lipids & Steroids	13	2	9	2	3	14	
Mineral & Bone	3					7	
Muscle	5	4		2			
Neuropharmacol.	20	4	2	35			
Neurophysiol.	10	9		3			
Nucleic Acids	8	2	14	2	2	2	3
Proteins	12		14		3	5	4
Radiobiology	3						
Renal & Water	10	7		4	7		
Respiration	9	12					
Toxicology	4			3			
Tumors & Cancer	6				9		6
Viruses	7				7		24
Vitamins	4					11	
Physiol. General	5	5					
Biochem. General	4		4				
Nutr. General	4					12	
Pharmacol. General	1						
Pathol. Movies	1						
Fed. Movies	1						

Read down. Example: Of the total attendance records of APS members, 17% were interested in cardiovascular sessions.

schedule for the programs. There were several complimentary remarks on the excellent management of the meetings by the Federation Staff and, considering the number of papers, the excellent programming.

4) Among general suggestions was a strong recommendation by many that abstract numbers appear in the program and that all morning sessions, regardless of society, be listed together. This is another indication of an apparent desire for integration.

FURTHER STUDY NEEDED TO ANSWER PERTINENT QUESTIONS

All of the compilations tabulated above represent the cooperative efforts of chairmen of sessions, members and non-member scientists who sent in postcards, and the Federation staff. The thanks of the Federation are extended to all of those who made this study possible.

The effectiveness of our present system for oral communication among scientists needs a careful study in depth. Mechanisms of scientific meetings for direct presentation of research findings have not changed greatly in essence over the past 50 or more years. There have, however, been some improvements and minor changes. Meetings have grown greatly in size and number with consequent complications, distracting attractions, and restrictions on the number of papers that can be presented. Difficulties in the so-called "corridor" personal contacts and exchanges have increased with the increasing number of scientific papers, to such an extent that persons having similar interests may sometimes be located in widely separated conference rooms. The basic need and desire of scientists to communicate with each other face to face has not changed, however, and has probably increased.

The size of the Federation meeting is certainly not due to member attendance, and the majority of papers presented are not presented by members. Many of these, however, have member co-authors, and all have member sponsorship. One question that arises is whether the annual meeting is primarily for the benefit of members or for non-members. while both should be served,

TABLE 10. *Chairman's Report**

Selected Sessions	Physiol.	Biochem.	Pharmacol.	Pathol.	Nutr.	Immunol.	Total
Physiology							
20 Gastrointest. absorp.	175	20	10	10	20	0	235
23 Hypothalamus	250	24	46	5	8	0	333
73 Active transport	350	50	3	0	0	0	403
128 Neurochemistry	20	40	10	0	0	1	71
131 Hypertension	80	15	20	12	1	0	128
187 Reproduction	75	30	30	10	5	0	150
241 Lipids: fatty acids	26	44	4	1	12	0	87
Biochemistry							
27 Peptide hormones	150	500	100	0	0	0	750
31 Mitochondria	20	100	20	0	0	0	140
139 Hormones and metab.	31	240	38	2	8	0	319
194 Lipid metabolism	17	167	3	0	6	1	194
201 Carbohydrate metab.	80	140	5	0	1	0	226
206 Lipid chemistry	14	194	8	2	7	0	225
Pharmacology							
38 Cellular and biochem. pharmacol.	4	15	62	2	6	0	89
96 Anesthetics	5	15	35	5	1	1	62
97 Drug metabolism	1	13	29	0	3	0	46
209 Renal pharmacology	15	4	66	0	0	0	85
263 Gastrointest. pharmacol.	12	10	15	0	0	0	37
Pathology							
43 Tissue response I	4	5	6	32	2	7	56
102 Tissue response II	0	23	5	32	4	0	64
105 Neoplasia	5	25	0	60	0	10	100
216 Hematology	5	22	1	19	3	4	54
264 Radiation	10	3	1	10	0	1	25
Nutrition							
47 Lipids and steroids	3	36	0	0	40	4	83
49 Vitamin B12	5	34	1	0	53	0	93
165 Nutrition: Gastroent. and path.	5	27	3	6	28	0	69
166 Symp.—Action of vitamin K	10	205	4	2	75	1	297
223 Symp.—Nutritional significance of selenium	3	47	3	5	175	0	233
Immunology							
52 Transplantation	4	16	1	2	0	86	109
169 Infection and resistance	4	6	2	10	0	58	80
170 Antigens and antibodies	10	50	0	10	0	300	370
225 Antibody formation II	0	10	0	50	0	300	360
226 Viruses III	1	20	1	7	0	150	179

* This table is based upon mid-session badge counts by the chairmen.

is the high percentage of non-member registration indicative of the fact that a great many probably qualified people enjoy benefits of the Annual Meeting without membership obligations?

Another question has been raised. Should non-members, except graduate students, be charged a higher registration fee than members? Non-members have practically all the privileges of members without the obligations of membership dues and journal subscription. The huge size of the Annual Meeting is due primarily to the large registration of non-members.

There is another question. Should areas of general Federation interest run all week in the central auditorium? There are about a dozen of these as shown in Table 9. Further analysis should show where a number of sessions of primary interest to two societies might be held in hotels that are situated close together.

Since the Federation is made up of research workers, it seems appropriate that some research should be done on the means of presenting research. The experimental design of a study might include the following specific aims:

1) Determine and analyze direct and contributory factors that have influenced the growth of the Federation's Annual Meeting and the Autumn meetings of two of its member societies.

2) Analyze past program and session structure, including programming and procedures, to organize others for an experimental trial period.

3) Utilize computer programming to determine the practical limits of their usefulness and the amount of intellectual effort needed at the input and output stages to supplement the work of the machine.

4) Develop categories and new classification to fit developing areas of research both for arrangement of papers in sessions and for classifying the abstracts of papers presented. This could include machine indexing of titles.

5) Study and make recommendations to the Federation Board and the Federated Societies on such matters as the Annual Meeting, possible regional scientific meetings, conference and panel type discussions, balance in the program between submitted 10-min papers and longer invited papers, and other matters of arrangements, facilities, and publications.

REPORTS TO THE FEDERATION

It is planned to publish at least on an annual basis in FEDERATION PROCEEDINGS results of these studies, to stimulate the interest and cooperation of the Federation membership and of scientists attending our meetings.

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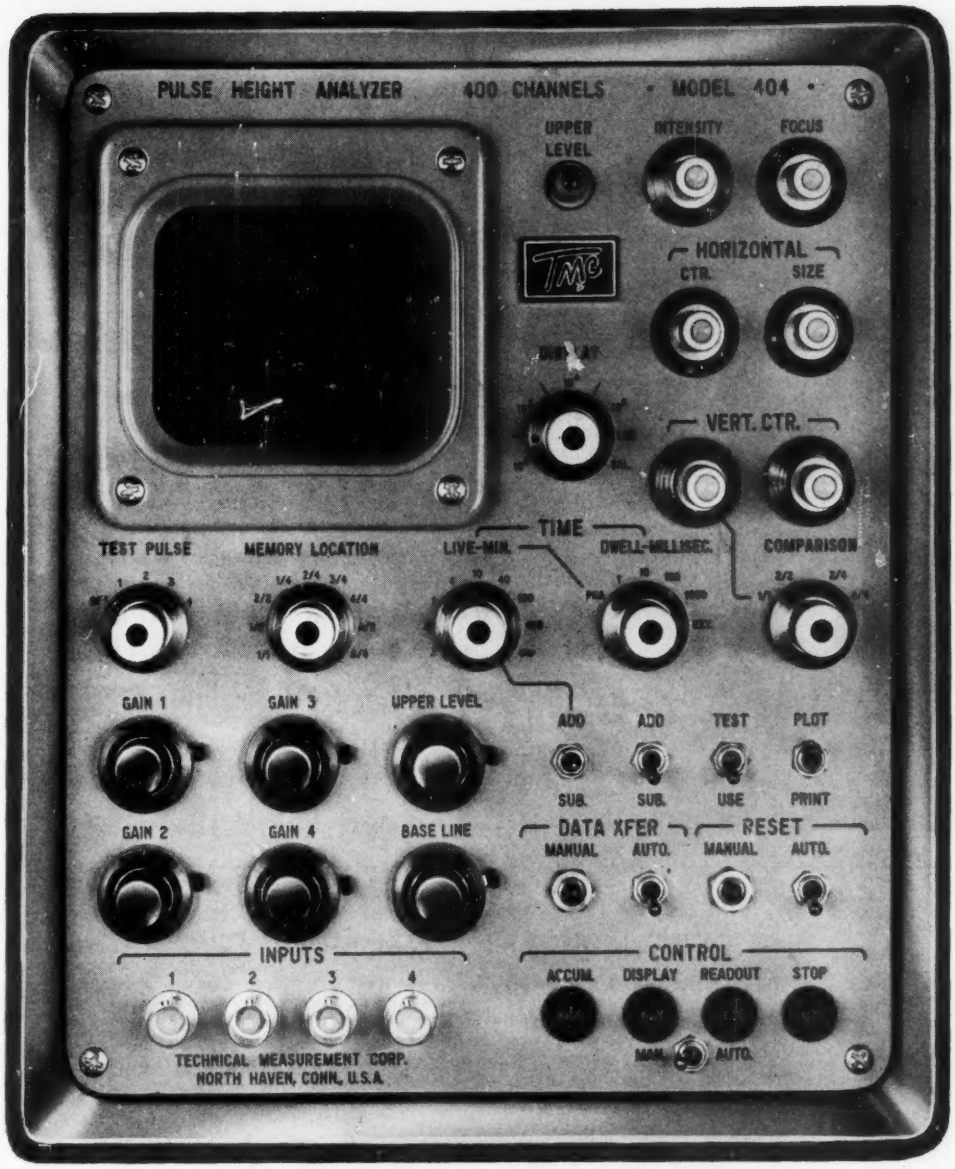

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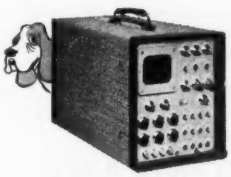

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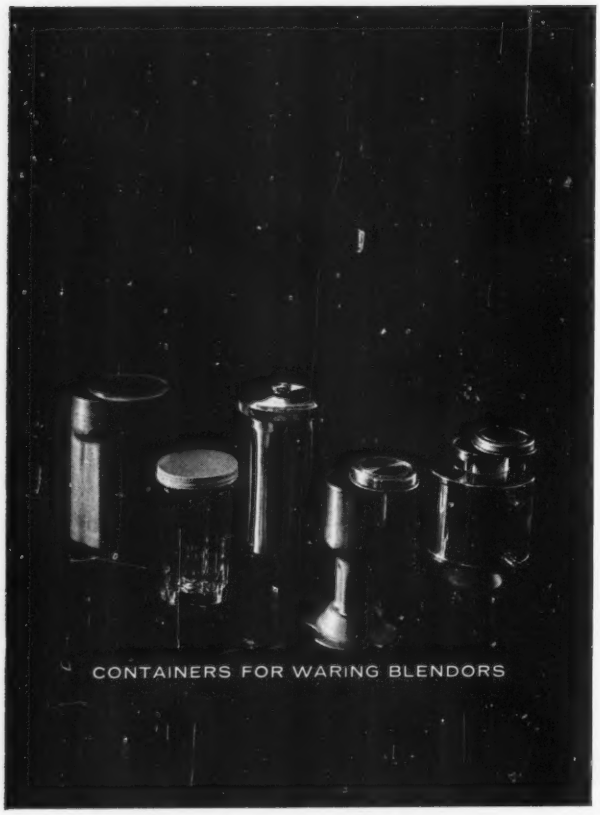

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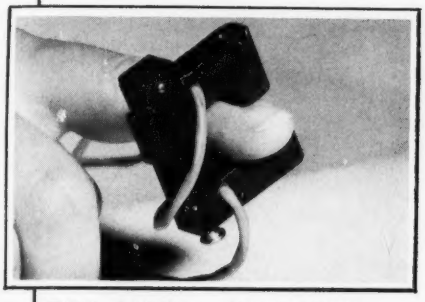

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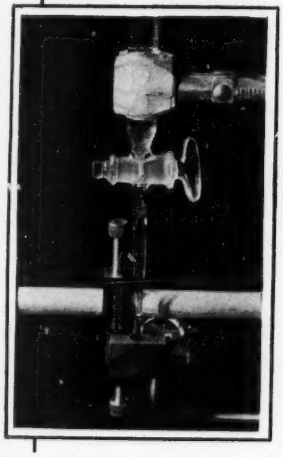

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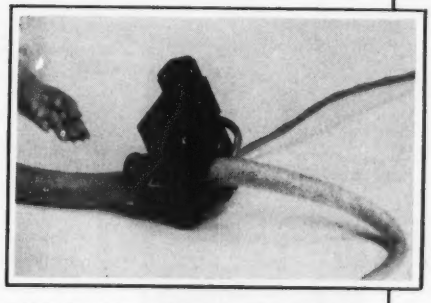

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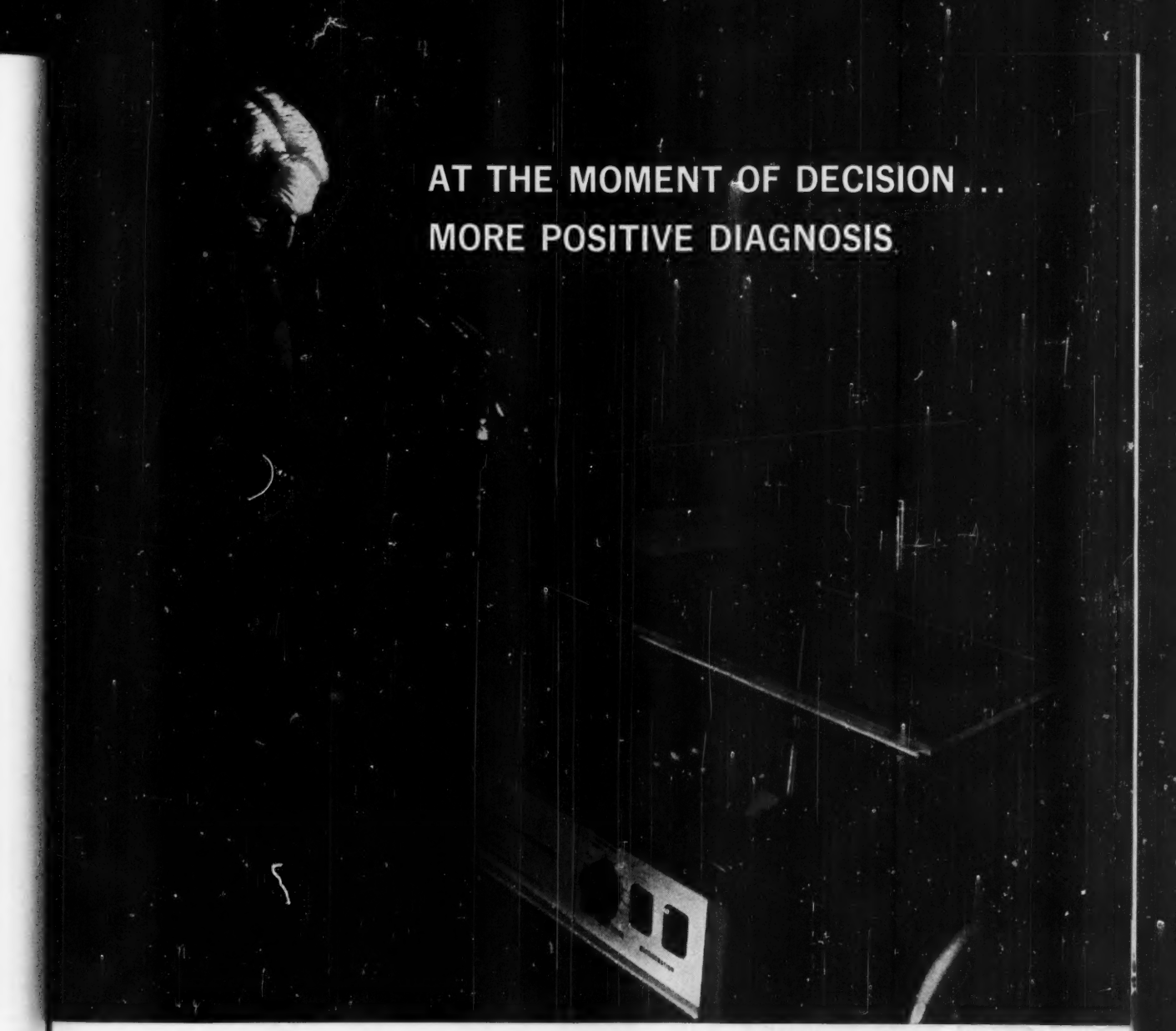

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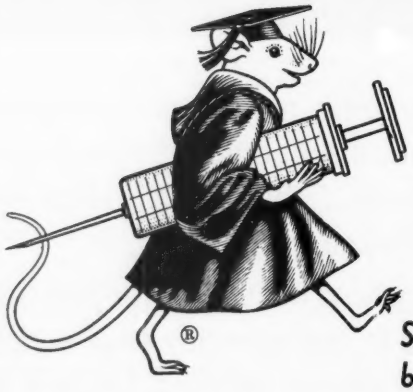

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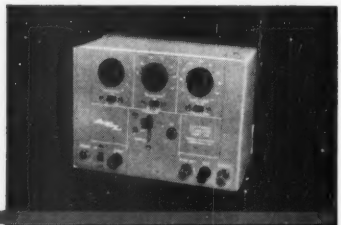

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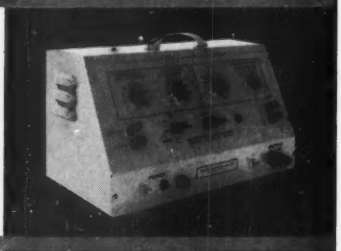

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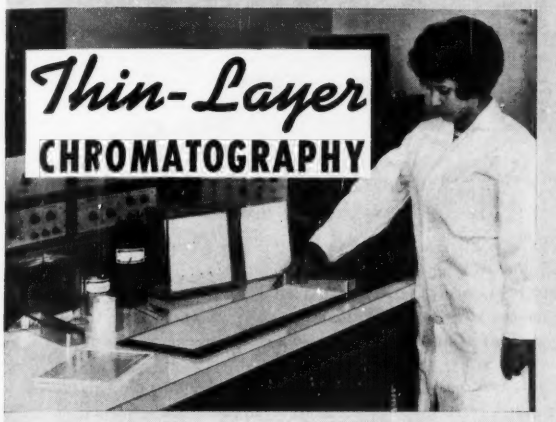

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Continued from page 78a

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In anticipation of the growing need for personnel under international programs in which it cooperates, the National Academy of Sciences-National Research Council is compiling a register of American scientists and other specialists who are interested in the possibilities of assignments abroad for periods ranging from several weeks to two years.

Persons who wish to be considered for any of such assignments are asked to fill out a special form, available upon request from the *Committee on International Exchange of Persons*, 2101 Constitution Avenue, N.W., Washington 25, D. C. Completion and return of the form will not constitute an application, but it would ensure a person's consideration for openings in his field.

Books and Journals Received

BASIC ESSENTIALS OF BLOOD GROUP THEORY AND PRACTICE. Kathleen E. Boorman and Barbara E. Dodd. Boston: Little, Brown & Co., 1961. 133 pp. $4.50

BIOELECTRICITY. E. E. Suckling. New York: McGraw-Hill Book Co., Inc., 1961. 233 pp. $8.75

BIOELECTROGENESIS. Editors: Carlos Chagas and Antonio Paes DeCarvalho. Princeton: Elsevier Publishing Co. (D. Van Nostrand Co., Inc.), 1961. 413 pp. $22.00

CONTINUOUS CULTURE OF MICROORGANISMS. S.C.I. Monograph No. 12. New York: The Macmillan Co., 1961. 272 pp. $8.50

DEVELOPMENT AND STRUCTURE OF THE CARDIOVASCULAR SYSTEM. Editor: Aldo A. Luisada. New York: McGraw-Hill Book Co., Inc., 1961. 225 pp. $9.95

FISH AS FOOD. Editor: Georg Borgstrom. New York: Academic Press, 1961. 725 pp. $24.00

FUNCTIONS OF THE BLOOD. Editors: R. G. Macfarlane and A. H. T. Robb-Smith. New York: Academic Press, 1961. 635 pp. $16.80

HEALTH AND FITNESS IN THE MODERN WORLD. A collection of papers presented at the Institute of Normal Human Anatomy, and the Ministry of Foreign Affairs, Rome, Italy: The Athletic Institute, 1961. 400 pp. $4.50

HEREDITY. A. M. Winchester. New York: Barnes & Noble, Inc., 1961. 269 pp. $1.75

HUMAN PITUITARY GONADOTROPINS. A Workshop Conference. Editor: A. Albert. Springfield, Ill.: Charles C Thomas, 1961. 434 pp. $16.75

LABORATORY MANUAL FOR GENERAL BIOLOGY, Rev. Ed. Willis H. Johnson, Richard A. Laubengayer, Louis E. DeLanney. New York: Holt, Rinehart and Winston, Inc., 1961. 181 pp. $3.50

PROBLEMS OF PULMONARY CIRCULATION. A. V. S. deReuck and Maeve O'Connor, Editors for the Ciba Foundation. Boston: Little, Brown & Co., 1961. 96 pp. $2.50

PROTEIN STRUCTURE. Harold A. Scheraga. New York: Academic Press, 1961. 305 pp. $8.00

RECENT ADVANCES IN BIOCHEMISTRY, 4th Ed. T. W. Goodwin. Boston: Little, Brown & Co., 1960. 301 pp. $11.50

Continued on page 82a

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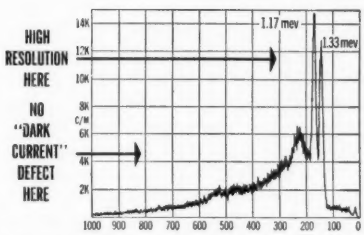
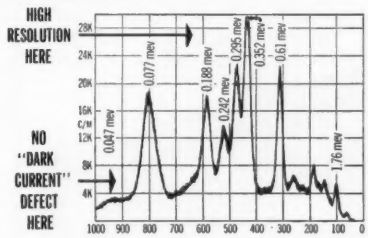


Actual spectrogram of Cobalt-60 made with a GSS-1 gamma spectrometer system. Window No. 3. Time constant: 2 sec.



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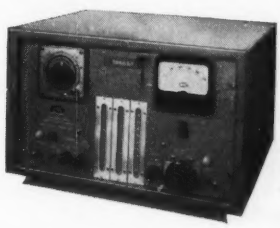

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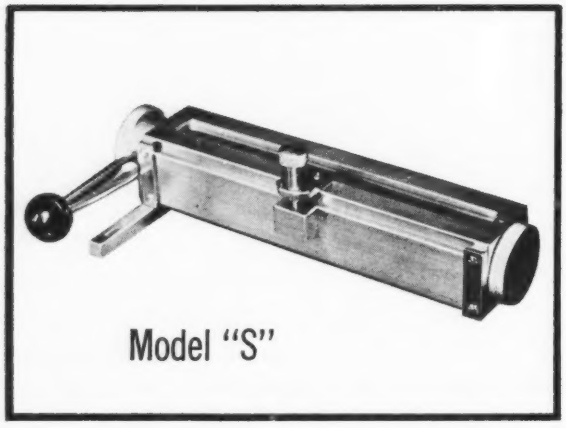

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Continued from page 80a

RESPIRATION IN HEALTH AND DISEASE. R. M. Cherniack and L. Cherniack. Philadelphia: W. B. Saunders Co., 1961. 403 pp. $10.50

THE MACHINERY OF THE BODY. Anton J. Carlson, Victor Johnson, H. Mead Gavert. Chicago: The University of Chicago Press, 752 pp. $6.50

Book Review

BLOOD AND OTHER BODY FLUIDS. PHILIP L. ALTMAN AND DOROTHY S. DITTMER, Eds. Washington: Federation of American Societies for Experimental Biology, 1961. xviii + 540 pp. $10.

This, the newest of the series of Biological Handbooks prepared by a Committee of the Federated Societies, presents a wealth of information concerning the blood and other body fluids. Data have been carefully collected from the literature concerning the volumes and composition of body water and its various subdivisions. An important section is devoted to the plasma proteins and non-protein nitrogen components of body fluids. Other sections deal with blood coagulation and blood group substances. Valuable information is included giving the composition and properties of urine, digestive secretions, reproductive secretions, milk, dermal secretions and the fluids of ear and eye. One of the more informative chapters presents information concerning body fluids during pregnancy. An outstanding virtue of this book is that it presents detailed information concerning body fluids in lower animals as well as in man. In addition to the commonly studied laboratory and domesticated vertebrates, information has been collected on a number of unusual and exotic species which will be of interest to the comparative physiologist.

The tables, diagrams, and figures are clearly organized and well annotated. References to original sources are readily available with each table or section. In controversial areas, the Committee has generally tried to follow an impartial course. In the tangled field of blood coagulation, this has led to the inclusion of five separate schemata illustrating the concepts of the leading workers in the field.

The role of the encyclopedist is thankless. No sooner has the ink dried on a publication of this type when important omissions and erroneous inclusions will be recognized. This book is no exception. For instance, detailed information is provided about the physical properties of albumin from four species of animals, but comparable data on human albumin have been omitted.

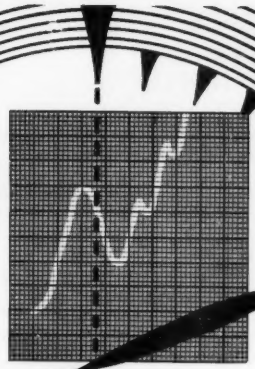
A similar omission is the failure to mention plasma transaminases in the tabulation of human plasma enzymes. With the exception of the phosphatases, no plasma enzyme is measured more frequently in laboratories of clinical chemistry.

A retrogressive step has been taken by the Committee in expressing the concentration of the important blood and serum electrolytes in terms of milligrams per 100 ml rather than milliequivalents per liter. The latter convention has been almost universally adopted by clinical physiologists since the fundamental contributions of Gamble, Peters and others during the 1920's and 30's.

Such minor criticisms in no way detract from the net worth of information packed into the 540 pages of this volume. It will be an indispensable part of any personal or institutional library dealing with the biologic and clinical sciences. Handbooks of this general type provide a partial answer to the vexing problem of making the vast store of information available to workers of all disciplines.

WILLIAM H. DAUGHADAY

A COMPLETE POLAROGRAM



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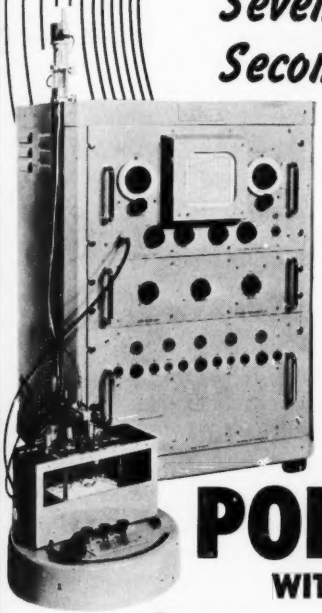

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M-NITROANILINE
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) *L. L. Uzman, M. K. Rumley and S. Van Den Noort, Nature, Vol. 186, No. 4724, pp. 559-560, May 14, 1961*

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) K. D. Miller, The Journal of Biological Chemis-, Vol. 235, No. 12, December 1960.

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MANN PROTEASE SUBSTRATE KIT No. 2080
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REFERENCES

(1) E. M. Press, R. R. Porter and J. Cebra, The Biochemical Journal Vol. 74, No. 3 pp. 501-514, 1960

(2) T. Webb and C. Lapresle, Nature, Vol. 188, No. 4744, pp. 66-67, Oct. 1, 1960

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REFERENCE

(1) A. W. A. Brown and A. G. Carmichael, Nature, Vol. 189 No. 4763 Feb. 11, 1961

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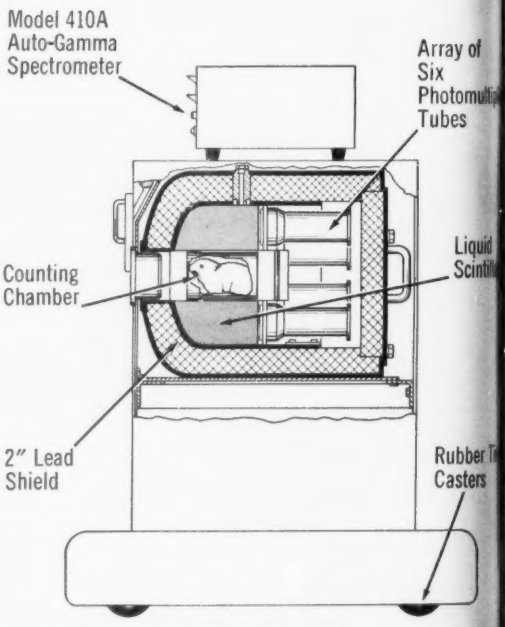

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